**Universidade de Aveiro** Departamento de Biologia
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# Doenças Bolhosas Autoimunes – Penfigóide Bolhoso, Pênfigo Vulgar e Foliáceo

# Autoimmune Blistering Diseases – Bullous Pemphigoid, Pemphigus Vulgaris and Foliaceus

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**Universidade de Aveiro** Departamento de Biologia
**Ano 2015/2016**

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# Autoimmune Blistering Diseases – Bullous Pemphigoid, Pemphigus Vulgaris and Foliaceus

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Mário Jorge Verde Pereira, Professor auxiliar do Departamento de Biologia da Universidade de Aveiro.

Dedico a minha dissertação à minha filha, a minha querida Matilde.

**o júri**

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**palavras-chave**

Doenças bolhosas autoimunes, penfigóide bolhoso, pênfigo vulgar, pênfigo foliáceo.

**resumo**

Penfigóide bolhoso e pênfigo pertencem a um grupo de doenças bolhosas auto-imunes que afectam tecidos saudáveis do corpo, causando formação de bolhas e lesões na pele. Penfigóide bolhoso é caracterizado pelo ataque aos queratinócitos basais, fazendo com que estes percam a capacidade de aderência à zona da membrana basal, enquanto no pênfigo os queratinócitos na epiderme e membranas mucosas perdem adesão celular.

Um único click no tópico “pemphigus” na PubMed revela mais de 9 000 artigos que providenciam informação acerca deste grupo de doenças bolhosas auto-imunes. Isto demonstra o enorme interesse clínico e científico em desvendar os mistérios que ainda cercam estas doenças, que começou há mais de 60 anos com a diferenciação de penfigóide bolhoso de pênfigo.

Penfigóide bolhoso é a doença bolhosa auto-imune mais comum e afecta maioritariamente os idosos. Tem uma taxa de mortalidade elevada sobretudo devido a terapia associada e outras complicações que surgem associadas à doença. As lesões são causadas por autoanticorpos que fixam o complemento, mediando um processo inflamatório. Pênfigo tem uma incidência superior nas mulheres. A idade média de início de pênfigo vulgar é entre os 60 e os 70 anos nos países Europeus e entre 30 e 50 nos restantes zonas do globo. Pênfigo foliáceo tem uma idade média de iniciação entre 30 a 45 anos. Além disso, as taxas de mortalidade de penfigóide bolhoso são cerca de duas vezes superior e de pênfigo cerca de três vezes superior, comparando com a população geral.

Muita informação aponta para uma clara predisposição genética da doença, combinada com factores que podem desencadear a doença. No que respeita o diagnóstico, a avaliação física é um marco importante, onde a pele é examinada, tal como as membranas mucosas e as unhas. Os pacientes também são submetidos a questões meticulosas sobre os sintomas, onde o historial médico não é deixado de fora. Examinação histológica quantitativa e qualitativa é sempre feita, por exemplo ELISA.

Hoje em dia os corticosteróides continuam a ser a terapia principal, contudo, novas estratégias terapêuticas têm vindo a ser desenvolvidas. A gestão terapêutica compreende uma serie de medicação, como os corticosteróides, micofenolato, rituximab ou abordagens inovadoras associadas a biotecnologia. O uso de compostos associados melhora o prognóstico do doente. Contudo, estas medicações podem levar ao aparecimento de complicações devido a alterações no sistema imunitário, tais como complicações respiratórias. Quando este tipo de complicações aparecem em ambiente hospitalar são, normalmente, devido a uma ou varias estirpes, o que dificulta o tratamento. Infecções com origem bacteriana, vírus, etc., são elas próprias associadas a penfigóide bolhoso e pênfigo. Tem vindo a ser documentados casos de ambas doenças associadas a outras complicações, como cancros, outras doenças do foro imunitário, neurológicas, etc.

Esta breve revisão aborda as três doenças bolhosas auto-imunes – penfigóide bolhoso, pênfigo vulgar e pênfigo foliáceo.

**keywords**

Autoimmune blistering diseases, bullous pemphigoid, pemphigus vulgaris, pemphigus foliaceus.

**abstract**

Bullous pemphigoid and pemphigus belong to a group of autoimmune blistering diseases that attacks the body healthy tissue, causing blisters and erosions on the skin. Bullous pemphigoid is characterized by an attack to the basal keratinocytes, making them to lose adhesion to the basement membrane zone, whereas in pemphigus happens that keratinocytes in epidermis and mucous membranes lose cell-to-cell adhesion.

A single mouse click on the topic pemphigus in PubMed reveals more than 9 000 articles providing one or the other information about this group of autoimmune blistering disease. This could only demonstrate the enormous scientific and clinical interest in unraveling the mysteries that still surrounds these diseases, which began over 60 years ago with the differentiation of bullous pemphigoid from pemphigus.

BP is the most common ABD affecting mainly the elderly. It has a high mortality rate, mainly due to therapy and some other complications disease-associated. Lesions are caused by autoantibodies that fix the complement and thus mediate an inflammatory process. Pemphigus has an increased incidence in women. PV has a mean age of onset between 60 and 70 years old in European countries and between the ages of 30 and 50 in the remaining countries of the world. PF has it between 30 to 45 years old. Moreover, the mortality rates of BP patients are two times higher and of pemphigus patients three times higher, than general population.

Much information points out a clear genetic predisposition for disease, combined with triggering factors. Regarding the diagnosis, the physical evaluation is a milestone, where the skin, mucous membranes and the nails are examined. Also, patients are submitted to meticulous questions about symptoms where the medical history is not left out. Some quantitative and qualitative histologic examination is always performed, like ELISA.

Nowadays, corticosteroids are still the main therapy; however, novel therapeutic targets have been developed.

The therapeutic management comprised a series of drugs, like corticosteroids, mycophenolate mofetil, rituximab or innovative approaches associated to biotechnology. Using two associated drugs really improved patients' prognosis. However, these medications could lead to the arising of other disorders, namely respiratory ones, as they debilitate the immune system. The contraction of such disease in a hospital environment often occurs due to one or more strains, which makes it difficult to choose a suitable therapy. Infections with bacterial origin, viruses, etc., also associate themselves to the diseases, sometimes. There have been documented cases of patients with pemphigus or BP associated with other disorders, such as neoplasia, neurologic disorders, other immune disorders.

This brief review will focus on three autoimmune diseases – BP, PV and PF.

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**Acronyms:**

ABD – Autoimmune blistering disease
Abs – Antibodies
ABSIS – Autoimmune Bullous Skin disorder Intensity Score
ACD – Allergic contact dermatitis
Ach – Acetylcholine
AH – Arterial hypertension
APD – Antibody Phage Display
APC – Antigen – Presenting Cells
AZA – Azathioprine
BAFF – B-cell activating factor
BMZ – Basement membrane zone
BP – Bullous Pemphigoid
BP180 – Transmembrane protein
BP 230 – Transmembrane protein
BPAG1 – Transmembrane protein
BPAG2 – Transmembrane protein
BPDAI – Bullous Pemphigoid Disease Area Index
CAR – Chimeric antigen receptor
CARR – chimeric autoantibody receptor
C3, C4, C5 – complement components
CD – T cells
CM – Corticosteroid hormone
CMV – cytomegalovirus
Col – Collagen
COL17 – Collagen XVII
CXCL – C-X-C motif chemokine
DEJ – Dermal epidermal junction
DHEAS – Dehydroepiandrostone sulfate
DIF – Direct immunofluorescence
DIBP – Drug-induced bullous pemphigoid
DP – Desmoplakin
DPP – dipeptidyl peptidase
Dsc – Desmocollin
Dsc3 – Desmocollin 3
Dsg – Desmoglein
Dsg1 – Desmoglein 1
DST – Dystonin gene
DTD – desmoglein-specific terminal domain
EA – Anchor domain
EBV – Epstein-Barr virus
EC – Anchor domain
ECD – Extracellular core domain
ECP – Eosinophil cationic protein
EGF – Epidermal growth factor
EGFR – Epidermal growth factor receptor
ELISA – Enzyme-Linked Immunosorbent Assay
EPF – endemic pemphigus foliaceus
ER – Endoplasmic reticulum
Fab – Fragment antigen-binding
Fc – Fragment crystallisable
FcγR – Low-affinity Fcγ receptors

FcεRI – High-affinity immunoglobulin E receptor
FcR – Protein found on the surface of certain cells
FK-506 – Tacrolimus
FS – Fogo selvage
GA – Garlic Acid
H-CDR3 – Heavy Chain Complementary – determining
H&E – Hematoxylin-eosin staining
HHV – Human herpesviruses
HIV – Human immunodeficiency virus
HLA – Human leukocyte antigen
HPLC – High-performance liquid chromatography
Hsp27 – Heat shock protein 27
HSV – herpes simplex virus
IA – intrecellular achor domain
IFN-γ – Interferon gamma
IgE – Immunoglobulin E
IgG – Immunoglobulin G
IIF – Indirect immunofluorescence
IL – Interleukin
ILD – Interstitial lung disease
IPL – Intracellular proline-rich linker
IVIg – Intravenous Immunoglobulin
KIF – Keratin intermediate filament
LD – Lamina densa
LL – Lamina lucida
LMJ11 – Sand salivary gland antigen
mAbs – Monoclonal antibodies
MAPK – Mitogen-activated protein kinase
MBP – Major basic protein
MC – Mast cells
MCP-4 – MC-specific serine protease
MCW – Antigenic sites clusters NC16A BP
MetS – Metabolic Syndrome
MHC – major histocompatibility complex
MMF – Mycophenolate Mofetil
MMP – Matrix metalloproteinases
MT-ATP8 – Mitochondrially encoded ATP synthase 8
MTX – Methotrexate
NE – Neutrophil elastase
OCT – Optimal perilesional area
PAM – Pemphigoid associated with malignancies
PAS – Periodic acid-Schiff
PDAI – Pemphigus Disease Area Index
PDT – Photodynamic therapy
PF – Pemphigus Foliaceus
PG – Plakoglobin
PKP-1 – Plakophilin-1
PV – Pemphigus Vulgaris
PV – sIVIg – PV specific intravenous immunoglobulin
PVA – Pemphigus Vulgaris Antigen
PVAS – Pemphigus Vulgaris Activity Score
PG – Plakoglobin
RA – Rheumatoid Arthritis

RCM – Reflectance confocal microscopy
ROS – Reactive Oxygen Species
SGLL – Sand salivary gland antigen
SLD – Sublamina densa
SLE – Systemic Lupus Erythmatous
SNP – Single nucleotide polymorphism
TA – Tannic Acid
TF – Tissue factor
TNF – Tumor necrosis factor
tPA – Tissue plasminogen activator
TPMT – Thioprine methyl transferase
TSC – Tuberous sclerosis complex
UV – ultraviolet
UVA – ultraviolet A
UVB – ultraviolet B
VEGF – Vascular endothelial growth factor
VL – Variable Light
VH – Variable Heavy
VH1-46 – Dominant Immunoglobulin Heavy Chain Gene

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# Chapter I

## Introduction to Characterization

This dissertation emerged not only due to my master degree but also from the necessity to gather information about the scourge that these autoimmune blistering diseases (ABD) are in our society. In this document it will be possible to analyse the lack of information that still haunts this theme, which is the main disadvantage for those who suffer from these conditions.

This document was structured in an intuitive way, easy to read, taking into account the large amount of information that it is gathered. So, it was divided in ten chapters that were selected in a conscious way. The first three chapters, excluding this one, intend to elucidate on the molecular mecanisms underlying the three diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV) and pemphigus foliaceus (PF). The fifth chapter guides us through diagnosis lay-outs, where it's possible to read about assays and come outs. I found important that this chapter came after the molecular basis because the assays also had a molecular basis. The following chapter, the sixth, describes the diseases that are associated to the three ABD. Then, I found it pertinent to talk about the triggers that could initiate the pathomechanisms underlying the three diseases. The genetic susceptibilities are described in chapter eight, where it is possible to read about gene alleles that may cause susceptibility to these diseases. Next I approached the treatment used in these diseases and its many side effects. And, lastely, the epidemiology data is described in the tenth chapter.

This review stands out from the others already published for the simple reason that this one covers all the most important topics, while the other reviews only covers few topics at a time. They are reliable and consistent, but in order to gather a comprehensive understanding of the big picture, you'd need to read many.

The ABD addressed in this document, as the name suggests, is characterized by an attack to the skin, more specifically to the epidermis. The epidermis is composed by the five layers (Proksch et al., 2008) demonstrated in the figure below (**Figure 1**): stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, stratum basale.

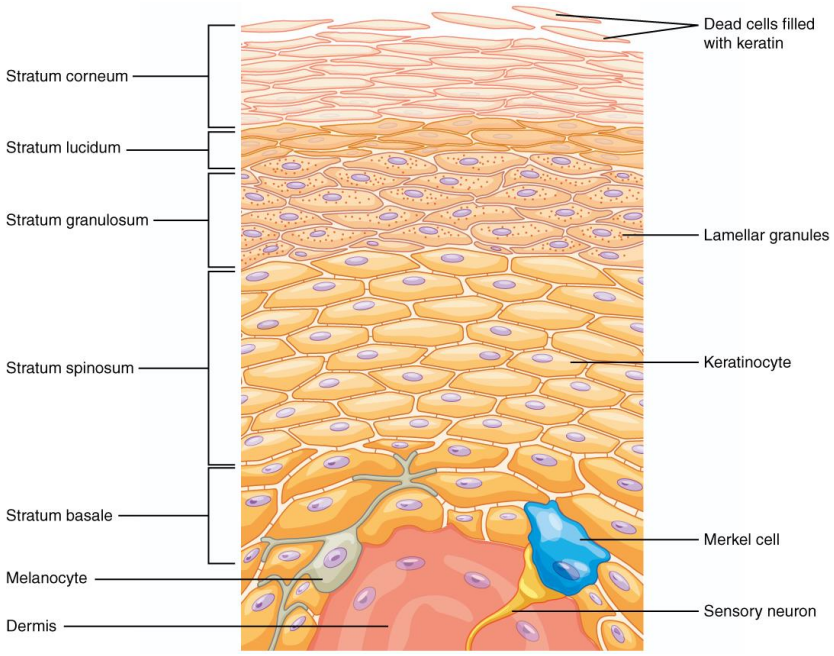


**Figure 1 – Layers of epidermis** (https://opentextbc.ca/anatomyandphysiology/chapter/5-1-layers-of-the-skin/)

While in pemphigus cases the attack occurs throughout the epidermis (DiMarco, 2016), in bullous pemphigoid (BP) cases the attack occurs exclusively in the basement membrane zone (Beutner and Jordon, 1964), between the stratum basale and the dermis.

### 1.1. Characterization

The ABD are characterized by a heterogeneous group of disorders that attacks the body healthy tissue, which will cause blisters and erosions that primarily affect the skin and mucous membranes. This group of diseases is chronic and it's important to distinguish from others non-autoimmune blistering diseases that have short duration and have typical clinical features, like herpes simplex, poison ivy, or bullous erythema mulfiforme. Mortality and significant morbidity are factors that have direct association with these diseases (Cotell et al., 2000; Schmidt and Zillikens, 2011). Despite being relatively uncommon, they are potentially fatal associated with deposits of autoantibodies and complement against distinct molecules of the epidermis and dermal/epidermal basement membrane zone (BMZ) (Velez et al., 2013).

### 1.2. Bullous Pemphigoid

The most common ABD is bullous pemphigoid, which mainly affects older people. The reported incidence of BP in Europe has more than doubled, in the past decade (Schmidt and Zillikens, 2013) and, despite the fact that a higher incidence occurs in the elderly, there are a few cases occorring in childhood (Reis-Filho et al., 2013), with a mean age of onset of 4.5 months old and being often severe with blisters in hands and feet. In terms of pathogenicity and diagnostic criteria, BP behaves similarly between adults and children (Schwieger-Briel et al., 2014). For instance, in Israel the incidence of BP in such young age as an incidence of 2.36 cases per 100 000 per year but in most world countries there

are no registry and the disease can easily be under-recognized (Waisbourd-Zinman et al., 2008).
Being a subepidermal autoimmune blistering disease, BP has autoantibodies that attack the hemidesmosomal structural proteins; wich will cause the cleavage between the epidermis and the dermis, as seen in **Figure 2** (Schmidt and Zillikens, 2011).
The clinical spectrum of BP includes tense blisters, urticarial plaques and prurigo-like eczematous lesions (Velez et al., 2013). When a patient suspecting BP searchs for some medical evaluation, the procedures must include histopathology that shows subepidermal blisters with a perivascular and an infiltrate made of eosinophils (Samhaber et al., 2016).

**Figure 2 – Tense blisters in a BP patient (adapted from Hammers and Stanley, 2016).**

### 1.3. Pemphigus Vulgaris

Another well know ABD is pemphigus. Here the two basic forms are covered: pemphigus vulgaris (PV) and pemphigus folieaceus (PF). The major incidence of PV is localized in Europe and USA, whereas the incidence of PF is mostly frequent in some areas in Brazil, along with other underdeveloped areas of the world (Aboobaker et al., 2001), like, Tunisia and Colombia (Tron et al., 2005).
Pemphigus vulgaris is an uncommon and chronic vesiculobullous disease that represent the majority of all pemphigus cases, with an average incidence of 0.1 – 1.0 cases per 100 000 per year. Moreover, prevalence of PV is predominant in regions with Jewish population. It is a potentially letal disease affecting mainly mucous membranes and skin. Females are the most affected gender, and have a mean age of onset of 50 to 60 years old. This disease usually has a Nikolsky sign positive (Velez et al., 2013).
The first thing to do when there is suspect of PV in a patient is observe if there are any mucocutaneous erosions or blisters, like in **Figure 3**. Then the oral mucosa should be carefully observed because is most affected in this disease (Kumar et al., 2016). Usually, PV mantains confined to mucosal surfaces. In cases that it is not confined, it extends to the skin, with a latency period of about 4 months. PV, usually, has a peak of incidence in the third to sixth decades of life (Harman et al., 2003). It causes extensive and painful erosions in mouth which could result in a decrease in food or drinks intake, although, esophagus, conjunctiva, nasal mucosa, vagina, penis, anus and labia may be affected too (Velez et al., 2013).
Before cortisteroid therapy become avalible in the 50s, PV held a high mortality rate, due to adverse effects of therapy (Bystryn and Steinman, 1996; Meurer, 2012).

**Figure 3 – Patient with erosions from PV (adapted from Hammers and Stanley, 2016).**

### 1.4. Pemphigus Foliaceus

Clinically, PF was first described by Dr. Pierre Louis Alphée Cazanave, in 1844. It was characterized as a disease that produces superficial cutaneous blisters and erosions. Dr. Pierre Cazanave described the non-endemic form occurring world wide and with a low incidence (Cazanave, 1844). The phenotype of this disease includes flaccid bullae which then ruptures and results in inflamed and excoriated skin, as seen in **Figure 4**, and it also affects the mucosae (Velez et al., 2013).

Diagnose is based on clinical manifestations like flaccid blisters and erosions of skin, on histologic findings, such as epidermal acantholysis, and on immunological abnormalties (Castro and Proença, 1982; James et al., 2011).

Usually PF affects both sexes and commonly manifests in the fourth and fifth decades. Desmoglein 1 (Dsg1) is the main disease antigen; however, others should also be taken into account, such as desmoplakin, periplakin and envoplakin (Velez et al., 2013). The endemic pemphigus foliaceus (EPF) is historically also called *fogo selvagem* (FS), in Brazil (Velez et al., 2013). Brazilian studies reported that serum concentrations of specific antibodies directly correlated with disease extent and activity; however, FS' healthy relatives may also have similar non-pathogenic autoantibodies. EPF was also described in Tunisia and Colombia (Velez et al., 2013). This disease can be triggered by some environmental factors, such as sunlight; parasites, such as *Trypanosoma cruzi*; and insects, like bed bugs and sand flies (Hans-Filho et al., 1999; Kano et al., 2000). When compared with PV, EPF is less aggressive and the therapy applied is also soft, but the generalized cases are more difficult to treat and eventually lead to high morbidity and mortality. While PV is more common in the age group of 61 to 75 years old, PF is more common between 30 and 45 years old (Khondker et al., 2014).

**Figure 4 – Patient with lesions from PF (from Hammers and Stanley, 2016).**

A study was made to evaluate the perception that general population have of pemphigus vulgaris (PV) and pemphigus foliaceus (PF), with a group of 115 subjects. About 14% in the case of PV and 6% in the case of PF, consider having those conditions worse than death. However, none in the cohort have the disease or was ever diagnosed with any form of pemphigus (Rencz et al., 2016).

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# Chapter II

## Mechanisms of the disease and Molecular Basis: Bullous Pemphigoid

### 2.1. BP230 and BP180 BP's autoantigens

In 1953, Lever first distinguished BP from pemphigus, through clinical and histological characteristics of both diseases (Lever, 1953). Later, in 1964, Beutner and Jordon proved that patients with BP produce circulating autoantibodies directed against the BMZ of the skin, using immunofluorescence (Beutner and Jordon, 1964).

BP is the most common of the pemphigoid diseases group, and is the most frequent autoimmune blistering disease in general. Within the group of the autoimmune disorders, pemphigoid diseases are a group characterized by autoantibodies against structural proteins of the dermal epidermal junction (DEJ) (Kasperkiewicz and Zillikens, 2007).

BP patients have IgG autoantibodies circulating against two hemidesmosomal antigens with 230 kDa – BP230 or BPAG1 – and with 180 kDa – BP180 or BPAG2 or type XVII collagen (COL17) – and these two proteins are components of hemidesmosomes, responsible for the connection of cytoskeleton of basal keratinocytes to structures of the papillary dermis (Mutasim et al., 1985; Stanley et al., 1981). The hemidesmosomes compose the epithelia multiprotein complexes, and are responsible for promoting the adhesion of epithelial cells to the underlying basement membrane. The major component of hemidesmosomes is α6β4 integrin, mainly responsible for transducing signals from the extracellular matrix to the inner cell. This communication modulates the cytoskeletons' organization, proliferation, apoptosis, and differentiation. A contact to anchoring fibrils is achieved via filamentous proteins, such as laminin 5. This contact has origin in the lamina densa of the basement membrane and extends into the subjacent connective tissue, and will terminate at structures known as anchoring plaques, which the major component is COL17, represented in **Figure 5** (Borradori and Sonnenberg, 1999).

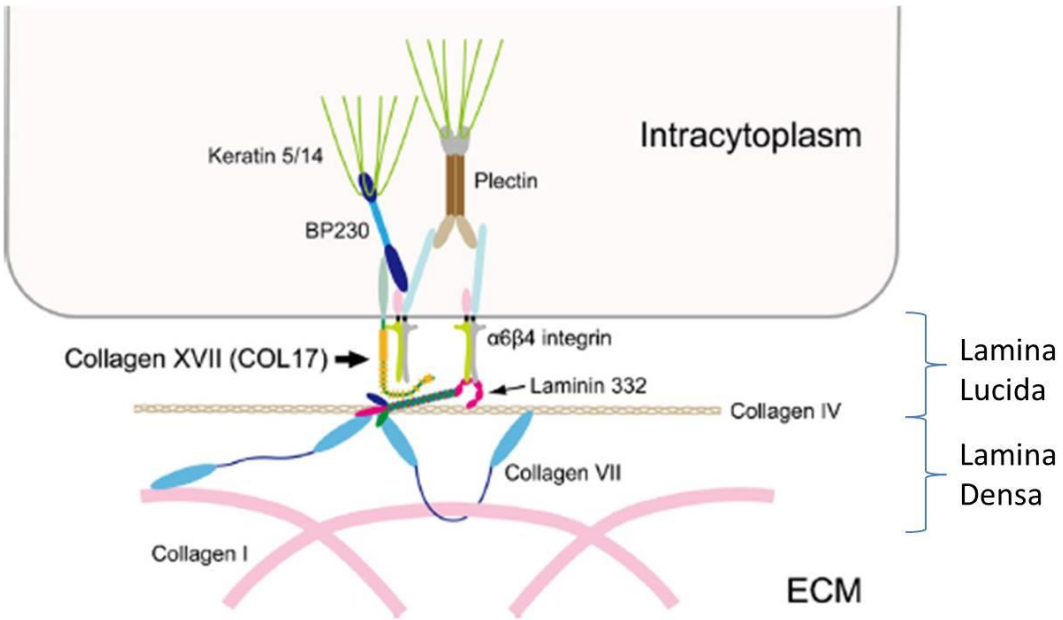


**Figure 5 – Molecular composition of hemidesmosomes (adapted from Nishie et al., 2014).**

Both BP180 (COL17) and BP230 have already been cloned and well characterized. This cloning has shown that corresponding epidermal cDNAs are products of distinct and unrelated genes (Diaz et al., 1990; Giudice et al., 1992; Hopkinson et al., 1992; Li et al.,

1992, 1993; Stanley et al., 1988). BPAG2 gene – BP180 – was mapped by chromosomal *in situ* hybridization to the long arm of human chromosome 10, at locus 10q24.3 (Li et al., 1991), whereas BP230 is a cytoplasmic component of hemidesmosomes that belongs to the plakin family of cytolinkers, promoting the association between hemidesmosomes and keratin intermediate filaments (Sawamura et al., 1991). BP230 also has a relevant homology with plectin and desmoplakins I and II (Green et al., 1992).
On the other hand, COL17 is a transmembrane protein with type II orientation, its amino-terminal region is localized in the intracellular hemidesmosomal plaque and its carboxyl-terminal region projects itself through extracellular milieu of the basal membrane zone (Giudice et al., 1991), as seen in **Figure 5**. It is molecule with a globular head (intracellular domain), a central rod and a flexible tail (Hirako et al., 1996). The extracellular region has fifteen collagen domains that alternate with noncollagen sequences, and its ectodomain contains numerous peptide segments in which every third position encapsules a glycine residue and the proline content is elevated. This pattern suggests that this proteins domain assembles to a collagen triple helix (Li et al., 1993). Both BP180 and BP230 are diagrammed in **Figure 6**.

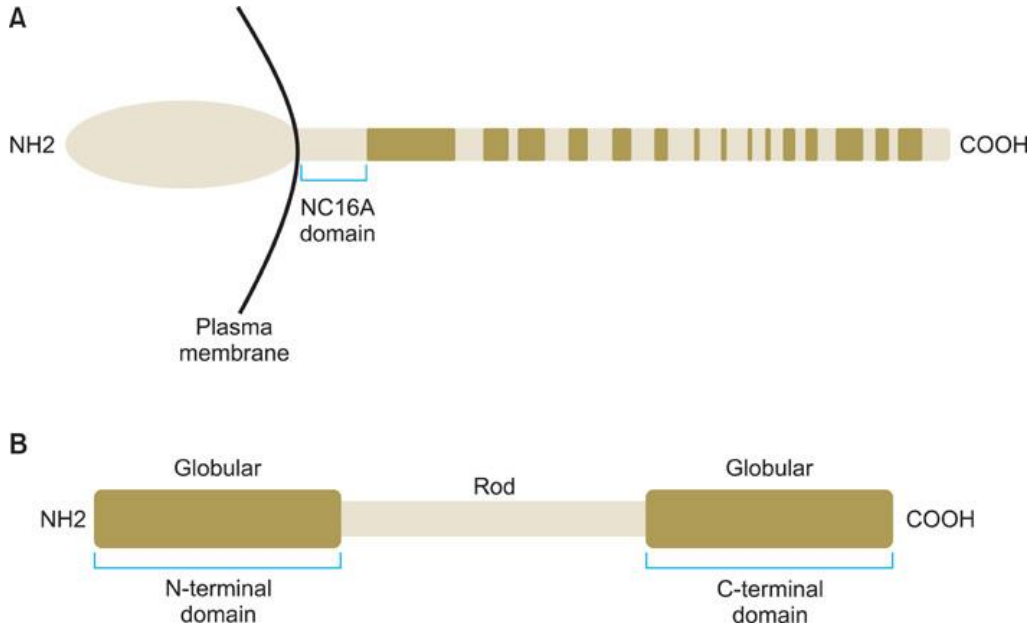


**Figure 6 – Diagram depicting BP180 (COL17) (A) and BP230 (B) (from Lee et al., 2012).**

Studies with immunoelectron microscopy indicate that the largest noncollagenous NC16A domain of COL17 is located inside the upper lamina lucida immediately subjacent to the hemidesmosome. This C-terminal region of BP180 co-localizes with the anchoring filaments at the interface between the lamina lucida and the lamina densa, as represented in **Figures 5** and **6** (Bédane et al., 1997; Masunaga et al., 1997). Also, is an easy target to pathogenic autoantibodies (Kasperkiewicz and Zillikens, 2007; Ujiie et al., 2010). It was demonstrated that a great number of BP patients with active disease recognized COL17 and others reacted with the ectodomain, a 120kDa protein that is shed from the keratinocyte surface (Schumann et al., 2000).
The importance of COL17 was also demonstrated by a truncating mutation in COL17A1 gene. This mutation compromised the assembly of hemidesmosomes, impairing both

mechanical stability of basal keratinocytes and dermo-epidermal adhesion. This information also reinforces that without the cytoplasmic domain of BP180, BP230, plectin and α6β4 are not quite effective in intermediate filaments conection to hemidesmosomes, so to extracellular matrix proteins (Fontao et al., 2004).

An enzyme-linked immunosorbent assay, utilizing baculovirus-expressed recombinant forms of $NH_2$ and COOH-terminal region of the BP180 ectodomain, screening sera from 116 patients with active BP was analysed. This test demonstrated that 80% and 47% of the BP patients recognized $NH_2$ and COOH-terminal, respectively. Also, patients with acute BP also had IgG1 antibodies against the $NH_2$-terminal region of the BP180 ectodomain, and patients in remission revealed higher levels of IgG4 autoantibodies. The IgG autoantibodies titers against the $NH_2$-terminal, and not against the COOH-terminus, could reflect the skin involvement. This event also demonstrates that the autoantibodies against NC16A BP'subdomains could critical in BP pathogenesis (Hofmann et al., 2002). This $16^{th}$ noncollagenous domain of BP180 – NC16A, represented in **Figure 6** – have four main antigenic sites clusters - MCW-0, MCW-1, MCW-2, MCW-3 (Zillikens et al., 1997). However, and despite the importance if this subdomain, BP autoantibodies are capable to recognize antigenic sites over the entire BP180 molecule (Di Zenzo et al., 2007; Perriard et al, 1999).

It is also important not to forget that BP patients also respond to BP230, the transmembrane component (Skaria et al., 2000; Tanaka et al., 1991). In fact, in the sera of BP patients, autoantibodies against BP180 are less detected than those against BP230, despite BP180 having autoantibodies epitopes in the extracellular domain. This data suggests that BP180 could be a target of BP autoantibodies, which could initiate the primary response, and then, BP230 autoantibodies will accelerate lesions (Ishiko et al., 1993).

Regarding BP230 recognition, it seems that some antibodies bound with the N-terminal half of the BP230 but in the majority of cases, the epitopes recognized by antibodies are mapped in the C-terminal region, represented in **Figure 6** (Skaria et al., 2000). Moreover, BP230 has more important functions besides composing the hemidesmosomes that are targeted BP autoantibodies. It also plays important functions in various organs, such as central nervous system and skeletal muscle, so the DST gene, encoding BP230, gives rise to three major isoforms: epithelial, neuronal and muscular (Künzli et al., 2015).

Since the correlation between epitopes of BP autoantibodies and the clinical features are not clearly elucidated, a recent study found a clear difference in autoantibodies profile, which divides BP in inflammatory and noninflammatory. In other words, it was concluded that the inflammatory phenotype, the more common, includes erythema and autoantibodies against the NC16A domain and the noninflammatory phenotype the autoantibodies seem to specificallty target the midportion of COL17 and not the NC16A subdomain. Also, the noninflammatory pattern reveals patients with a reduced erythema and short lesional infiltration of eosinophils (Izumi et al., 2016).

### 2.2. Immunity in Bullous Pemphigoid

Since human and murine BP180 antigens are not cross-reactive, Liu et al., in 2008, humanized a mouse strain expressing the BP immunodominat epitopes – BP180 NC16A – to study the humoral immune response in BP disease immunopathologhy. The

humanized NC16A mice injected with anti-BP180 NC16A autoantibodies developed BP-like subepidermal blisters and the mice pretreated with mast cell activation blocker or depleting of complement or neutrophils, become BP resistant (Liu et al., 2008). It was also demonstrated that BP patients recognize at least another antigenic reactive site other than NC16A, suggesting that in the course of the disease patients exhibit a distinct epitope pattern and that reactivity against various intracellular epitopes, besides NC16A, can be observed even in the early stages of the disease (Di Zenzo et al., 2004). This information can be validated by studies with animal models that have been suggesting that autoimmune diseases are characterized by an “early” phase, in which the immune response is restricted to one or two epitopes among the antigen (dominant epitopes) and a “late” phase, where specificity spreads to additional subdominant epitopes – epitope spreading (Chan et al., 1998; Vanderlugt and Miller, 2002). The evidence of epitope spreading phenomenon has been reported in humans too (Bonifacio et al., 2000; Tuohy et al., 1999). As mentioned above, NC16A domain is the leading pathogenic target in BP, and antibodies (Abs) that react against a small portion of NC16A are essential for inducing depletion of COL17. Along with this, Natsuga et al. proposed that, besides the complement-dependent pathway leading to inflammation, direct effects of pathogenic antibodies, which deplete COL17 in epidermal keratinocytes, might contribute to skin fragility in a complement-independent pathway (Natsuga el al., 2012). These pathogenic antibodies belong to the subclasses of the immunoglobulins IgG1 and IgG4 (Bernard et al., 1990; Sitaru et al., 2007; Zhou et al., 2016) and IgE isotype (Döpp et al., 2000). It also has been proven that IgE and IgG4 react against two different epitopes within the BP180 NC16A – MCW-1 and MCW-2. The major IgG subclass targeting BP180 NC16A before treatment are IgG4 and IgG1 and the IgE levels in sera and blister fluid are usually high, correlating the levels of these autoantibodies with disease activity (Döpp et al., 2000; van Beek et al., 2016). Usually, IgG4 is present in relatively low amounts in the sera of healthy individuals, its level increase upon repeated antigen exposure, which can be used as a marker of successful immunotherapy. Zuo et al., showed that IgG4 can function in an inhibitory capacity in autoantibody mediated autoimmune disease, by passive transfer. It was imperative to solve the mystery of why IgG4 anti-NC16A antibodies bind the pathogenic NC16A domain, but do not induce disease. Their results suggest that anti-NC16A IgG4 binds to NC16A domain and blocks or reduces pathogenic anti-NC16A IgG1 and IgG3, binding to the same sites, which cause the reduction of anti-NC16A IgG1 and IgG3 mediated complement activation and subsequent neutrophil infiltration and BP blistering. Administration of IgG4 anti-NC16A antibodies could represent a new therapy strategy (Zuo et al., 2016). Moreover, Mihai et al. reported that BP IgG4 autoantibodies have the ability to induce leukocyte-dependent tissue damage. After binding to the epidermal basement membrane, IgG4 autoantibodies recruit and activate leukocytes and induce dermal-epidermal separation in the cryosections of human skin. Nevertheless, comparatively to IgG1, IgG4 autoantibodies show a lower pathogenic capacity (Mihai et al., 2007).

A recent study reported results claiming that levels of anti-BP180 NC16A IgE generally reflect disease severity over its course but not early on. This conclusion emerged from previous results, that indicating levels of IgE were higher in the sera than blister fluids (Bing et al., 2015). It is also reaffirmed that IgE anti-BP180 antibodies have correlation with disease activity, but not with disease phenotype (Hashimoto et al., 2016). Hofmann et

al., in 2002, reported that in a sample with 116 patients with active BP, about 78% were IgE-reactive and had generalized skin involvement (Hofmann et al., 2002). IgE and IgG seem to target the same or similar epitopes of BP230 and BP180, which indicates a good relation correlation of IgE and IgG auto reactivity in classic BP (Fania et al., 2012). It remains a challenge to evaluate the vital roles of IgE in the BP development due to the low levels in circulation and the lack of easy tools for assays (Ujiie, 2015), giving rise to some inconsistencies concerning the IgE role. So, how do IgE autoantibodies to COL17 act in the development of BP? It is hypothesized that IgE autoantibodies bind to FcεRI that are expressed by eosinophils (Messingham et al., 2014[b]; Delaporte et al., 1996), mast cells (Dimson et al., 2003) and basophils in blood (Messingham et al., 2014[b]) or it binds to target antigen COL17 at the DEJ in the skin (Parodi and Rebora, 1992; Provost and Tomasi Jr., 1974; Yayli et al., 2011). The IgE and COL17 bound mast cells have been demonstrated in lesion of patients with BP (Dimson et al., 2003). All combined, the COL17 ectodomains seem to bind to IgE autoantibodies, which in turn will bind to mast cells or eosinophils, promoting degranulation, as previously proposed by Messingham et al. (Messingham et al., 2014[a]), and as demonstrated in **Figure 7** (Ujiie, 2015). The mast cell granulate released, such as histamine, induce vasodilation and increase vascular permeability, and cytokines – such as TNF and IL-6 – enhance leukocyte recruitment (Wernersson and Pejler, 2014), which results in the development of the inflammatory process. High levels of histamine in blister fluid from untreated patients with BP were reported to be 10 to 50 times higher than the blister fluid from other blistering diseases and about 100 times higher than the plasma histamine level of other patients and normal volunteers (Katayama et al., 1984). As we can see in **Figure 7**, the IgE autoantibodies to COL17 also can be internalized which leads to the number decreased of hemidesmosomes at the DEJ in organ-cultured skin in a FcR-independent manner (Messingham et al., 2011). Therefore, the direct effect of IgE autoantibodies on basal keratinocytes may contribute to weak adhesion strength at the DEJ. Still, the effect seems limited, because IgE autoantibodies alone fail to induce apparent dermal-epidermal separation in animal models (Fairley et al., 2007; Zone et al., 2007), and although some BP patients reveal IgE deposition at the DEJ, the disease severity doesn't seem to be different between IgE positive cases and IgE negative cases (Moriuchi et al., 2015), which, again, comes into confronters some mentioned studies about the influence of IgE in disease course.

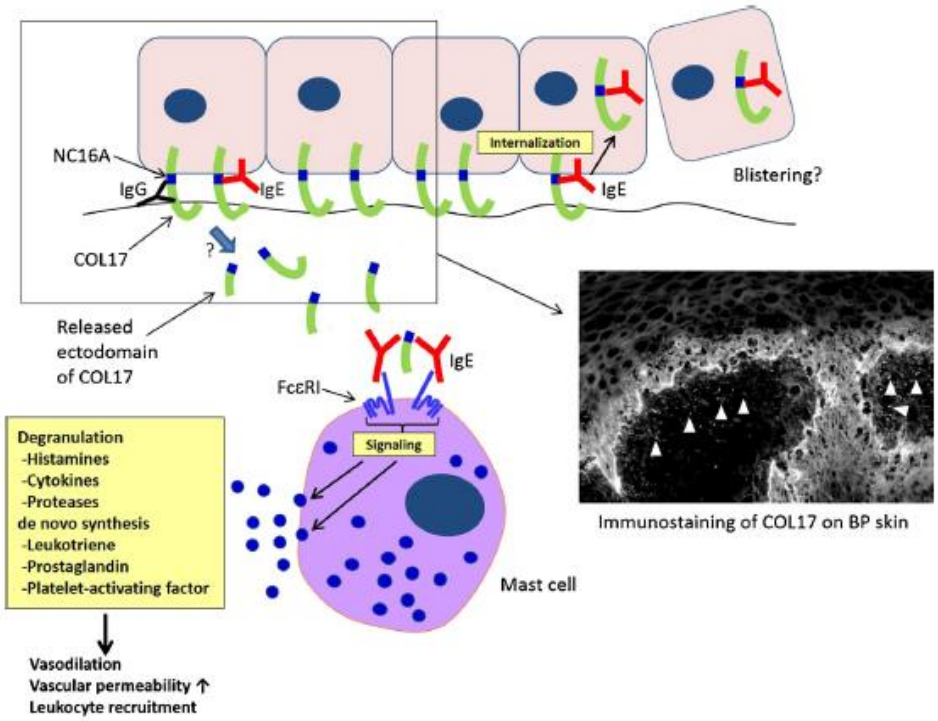


**Figure 7 – Proposed pathomechanism of IgE autoantibodies in BP. The COL17 ectodomains that are released bind to IgE autoantibodies mast cells and eosinophils in the dermis. After crosslink between FcεRI by IgE and the autoantigen, histamines and cytokines are released, which lead to a signalling cascade. Moreover, the IgE autoantibodies to COL17 could directly bind to COL17 on the basal keratinocytes, inducing internalization of the immune complex into cytoplasm. This phenomenon leads to a lack of COL17 and, consequently, a decrease in adhesive strength at DEJ (from Ujiie, 2015).**

A case report of a 67 years old Japanese man with BP with high levels of IgE anti-BP180 and anti-BP230 in the serum, and an unmanagenable clinical course, suggests that the high levels of IgE autoantibodies anti-BP180 play an important role in BP pathogenesis (Akasaka et al., 2016). Moreover, Ishiura et al. reported that about 30% of 67 BP patients had IgE anti-BP180 antibodies and 67% had IgE anti-BP230 antibodies. In this case, the IgE anti-BP230 antibodies seem to be strongly associated with local eosinophil infiltration (Ishiura et al., 2008). Iwata et al. corroborates this fact with a study where it was concluded that IgE anti-BP180 antibodies do relates with disease severity and activity of the disease (Iwata et al., 2008).

These entire findings together still demonstrate that the IgE anti-BP230 antibodies play an important role in BP pathogenesis; however, further studies are suggested.

When concerning the IgG, it was demonstrated that IgG autoantibodies to COL17 could deplete COL17 in cultured normal human keratinocytes (Iwata et al., 2009).

Li et al. affirmed the IgG1 is the pathogenic autoantibody in bullous pemphigoid, using a mice model (Li et al., 2010).

Over the years the attempts of passive transfer of blister-inducing autoantibodies in BP have been controversial, meaning that while some authors claim to be successful in this task, others seem to fail in it. For instance, Sams and Gleich transfused Rhesus monkeys with plasma from three BP patients and they did not have any success reproducing the features of BP, in any of the three animals (Sams and Gleich, 1971), while, in 1981,

Anhalt et al., demonstrated that the rabbit cornea could be used as a target tissue for BP autoantibodies. Results proved that intrastromal injection of BP autoantibodies reproduced the clinical, histologic, and immunologic features of bullous pemphigoid (Anhalt et al., 1981). Since then, many have been theories for the failure of some and the success of others. So, in the present year, Iwata et al. reviewed the animal model theme, in order to understand the evolution of these models that have such a high impact in the pathomechanisms of ABD. These models also play a crucial role in attesting novel therapies efficiency. The authors concluded that it is possible to come to an ideal animal model, mostly in mice. They also determined, through an exhaustive research, that there are four main pathways to induce spontaneous ABD, namely by transfer of autoantibodies, by transfer of autoantigen specific lymphocytes, by immunization or by genetic alterations (Iwata et al., 2016).

For instance, in 2015, Hurskainen et al., designed a genetically modified mouse model, deleting NC14A region, which corresponds to NC16A in humans. This deletion led to a decrease of the amount of COL17 in skin, but it did not prevent ectodomain shedding. Although there was no change in the phenotype, microscopically it was possible to observe subepidermal microblisters, rudimentary hemidesmosomes and the detachment of the BMZ. Also, eosinophila and high levels of IgE were registered. These ΔNC14A mice provide a reproducible BP-related mouse model with spontaneous disruption of self-tolerance and development of autoantibodies (Hurskainen et al., 2015).

### 2.3. Each Inflammatory Cell Plays Its Own Role

It is well known that the triggers underlying the autoimmune response in BP are not fully understood. Might BP be a result of a breakdown of peripheral tolerance to BP antigens? And would this mechanism be enough to induce autoantibody formation?

Thoma-Uszynski et al., demonstrated that the majority of thirty-five BP patients had autoagressive T and B cells autoantibody reactive with defined extracellular regions of BP180 and, to a lesser extent, of BP230. Knowing that NC16A region represents a major binding site for autoantibodies in bullous pemphigoid, they were able to conclude that NC16A region contains immunodominant epitopes for both autoaggressive T and B cells, however, this study stablished that autoreactive T and B cells also recognize epitopes located within the COOH and $NH_2$ terminal and central portion of the BP180 ectodomain. Summarily, this study defines that T and B cell recognition of the $NH_2$ terminus and midportion of BP180 seems to be associated with extensive and limited BP, respectively. T and B cell recognition of BP230 seems to be focused on the $NH_2$ and COOH terminal domains. Such observations led to the concept that BP180 is the primary autoantigen of BP (Thoma-Uszynski et al., 2006). T cells from BP patients were extracted to confirm their reaction to BP180. Also it is postulated that, not only T cells are reactive to BP180 as also the BP180-specific T cells are of the CD4 lineage (Lin et al., 2000).

In 2010, a case report mentions a patient that, by immunohistochemistry (IHC), revealed the presence of abundant CD45 lymphocytes, surrounding blood vessels, eccrine sweat glands and nerves. Also, this patient displayed IgG, IgE and fibrinogen antibodies against the BMZ (Abreu-Velez et al., 2010). This case alerts us to the possibility of the existence of more factors contributing to BP pathogenesis.

There is cooperation between B cells and $CD4^+$ Th cells, which is important for the production of antibodies by B cells. Self-antigens of the BMZ - BP180 and BP230 - in BP patients are caught by antigen-presenting cells, processed and bound to major histocompatibility complex class (MHC) II and displayed on the cell surface. The recognition of these epitopes by T cells will trigger the release of various cytokines and, consequently, B cells are stimulated to produce autoantibodies (Lo Schiavo et al., 2013).
It has been postulated that the imbalance between autoreactive Th and T regulatory cells (Tregs) is critical to the outbreak of BP disorder, and the difference between patients and normal individuals possessing the same autoreactive T cells consists in a deficit of the T regulating function (Grando, 2011). There is increasing evidence of dysfunctional Tregs in the regulation of the production of pathogenic autoantibodies in BP. Such studies suggest that Tregs may represent a great tool to specifically restore immune tolerance in autoimmune bullous skin disorders (Hertl et al., 2006).
Büdinger et al., reported that CD4 memory T cells exhibit a Th1 and Th2 mixed cytokine profile and that responses to BP180 were restricted by the BP associated HLA-DQB1*0301 allele in BP patients. Also, this T cells that are reactive to BP180, were found in healthy individuals. However, in the healthy individuals, Th2 cells are absent which strongly suggests that autoreactive Th2 cells responses to BP180 are restricted to BP patients and are crucial to pathogenesis (Büdinger et al., 1998). It is known that Th1 cells, mainly IFN-$\gamma$ that are found at an increased amount in sera and blister fluid of BP patients, are able to induce production of IgG1 and IgG2 and, for instance, Th2 cytokines, including Interleukin(IL)-4, IL-5 and IL-13, have been found to regulate the production of IgG4 and IgE. Detection of the three isotypes in BP patients, strongly suggests that both Th1 and Th2 cells are involved in regulation of the response to BP target antigens. Also, it's possible to conclude that BP stages can be defined through the Th1/Th2 balance. All these findings together leads to the belief that BP is a T cell dependent autoimmune disease with the presence of $CD4^+$ lymphocytes, mainly Th2, autoreactive T cells in the peripheral blood of patients that recognize the BP180 ectodomain (Giomi et al., 2002; Rico et al., 1999).
Moreover, in a previous study, Leyendeckers et al. 2003, detected and characterized BP180 specific $IgG^+$ B cells in eleven out of fourteen blood samples of BP patients (Leyendeckers et al., 2003). Also, Qian et al., demonstrated high levels of B-cell activating factor (BAFF) in memory B cells, specifically found intracellularly in BP patients. This abnormal BAFF production might disturb immune tolerance, allowing survival of autoreactive B cells and triggering autoimmune disease, such as BP. So BAFF expression in B cells might be relevant for BP pathogenesis (Qian et al., 2014). The depletion of B cells in some autoimmune disease boosted further investigation about the potencial of drugs that couyld possibly modulate the functions of B cells, for instance, in the treatment of some ABD (Browning, 2006).

### 2.4. Tissue Injury Mechanisms, Blister Formation and Fluids

The mechanisms that might cause BP autoantibodies to be pathogenic have been exhaustively studied and it could imply the complement activation, recruitment of inflammatory cells, liberation of proteolytic enzymes and direct interference with the adhesion function of the autoantigens.

### 2.4.1. When Complement System Plays a Role: Step 1

Gammon et al., demonstrated the relevance of leukocytes in BP, using serum samples from BP patients incubated with cryosections of normal neonatal human foreskin, obtained by routine circumcision of healthy neonates. This interaction causes a chemotaxis mediated by pemphigoid antibodies and fresh serum which led to a leukocyte migration to DEJ, and this event culminates in the separation of this junction. It was shown that this leukocyte chemotaxis and attachment to the junction requires the complement activation via classical pathway and evidence the C5 role (Gammon et al., 1981). Moreover, it was demonstrated that C5a can interact with mast cells and thus trigger the p38MAPK cascade. This cascade will cause the mast cells degranulation, also crucial for blistering (Heimbach et al., 2011).

There are some studies that demonstrate that BP autoantibodies fix complement *in vitro* and *in vivo*, via classical and alternative pathway, and that C3 and C5 are detected by immunofluorescence along the BMZ of perilesional skin in almost all patients with BP (Naito et al., 1984; Provost and Tomasi Jr., 1973). Also, it was demonstrated that both BP autoantibodies and C3 can be detected at the site of the injury, the lamina lucida of the skin (Schmidt-Ullrich et al., 1975). It seems that most serological positive BP sera fix complement, via both classical and alternative pathway (Jordon et al., 1985). Once again, the role of the complement system activation is emphasized through the observation of the levels of total hemolytic complement and of individual complement components in blister fluid from BP patients, which are lower than those found in control blister fluids in the sera of these patients (Jordon et al., 1973). In 2016, a statistical analysis revealed the importance of complement activation. A group of 300 BP patients were studied and about 83.1% showed C3 deposition along the epidermal BMZ, in their skin biopsy (Romeijn et al., 2016). Liu et al. used a mouse model to demonstrate the role of complement in the pathogenesis of subepidermal blistering, concluding that there is strong evidence that supports the idea that complement activation is crucial for the production of subepidermal blisters caused by anti-BP180 antibodies (Liu et al., 1995). So it was obvious that complement components are also present in the region of blister formation (Panelius and Meri, 2015).

In order to try to understand by which way the complement would be activated, in 2006, a study show the roles of the different complement activation pathways, through a passive transfer of antibodies to the murine BP180 (mBP180). So, this data demonstrated that activation of the classical pathway is crucial for disease development and that the alternative pathway acts in concert with the classical pathway in subepidermal blistering. This study points out an important fact, that the contribution of the alternative pathway to experimental BP usually is underestimated and that the implication of the lectin pathway cannot be fully discharged (Nelson et al., 2006).

But the controversy re-ignites by analyzing a case control study with two patients diagnosed with BP, reporting no complement activation at the BMZ. Direct immunofluorescence of the skin showed that IgG4 was the major subclass on the anti-BMZ IgG antibodies in both cases; moreover, complement fixation (CF) test demonstrated that circulating autoantibodies against BP180 from either case did not fix complement, whereas sera from common BP patients did. So, these findings suggest that there could

be an alternative mechanism complement-independent, in blister formation, making some cases complement-independent (Dainichi et al., 2016). Back in 2014, Ujiie et al. also demonstrated the involvement of complement-independent pathways, through a generation of C3-deficient COL17-humanized mice. It was proven that the deposition of Abs, and not complements, is important to the blister induction formation, both in neonatal and adult mice (Ujiie et al., 2014).

### 2.4.2. When Inflammatory Cells Also Interfere: Step 2

The autoantibodies react with an antigen located in the lamina lucida region of the basement membrane zone – anti-BMZ autoantibody – that characterizes bullous pemphigoid, triggering seems to be mediated by inflammatory cells (Jordon et al., 1985). Hereupon, among inflammatory cells that are able to mediate tissue injury, we have mast cells, neutrophils and eosinophils.

#### a. Mast cells Interference: Step 2.1.

It was 1978 when Wintroub et al. report the presence and degranulation of mast cells (MCs) in BP lesional sites (Wintroub et al., 1978). Such discovery was subsequently confirmed by further investigations. This degranulation of the mast cells was also seen in lesional skin of mice injected with pathogenic anti-mBP180 antibodies (Borrego et al., 1996; Chen et al., 2001; Dvorak et al., 1982). Antigen-specific degranulation of mast cells from BP patients suggests the mechanism by which IgE may contribute to lesion development (Dimson et al., 2003; Fairley et al., 2005).

Chen et al., ascertained that mice deficient in mast cells, macrophages or neutrophils were resistant to experimental BP, whereas in wild-type mice and T or B-deficient mice (or both T and B cells) pathogenic anti-murine BP180 antibodies triggered BP skin disease. Also, the reconstitution of the mast cells restores the pathogenic activity of anti-mBP180 IgG (Chen et al., 2002[b]).

It was also proven that complement activation is required to induce mast cells degranulation. For instance, C3a and C5a fragments are responsible for the mast cells degranulation induction. Also, the C5b deficiency completely eliminates mast cells degranulation (Chen et al., 2001, 2002[b]; Muller-Eberhard, 1988).

Mast cells are responsible for the production of many mediators such as leukotriens, platelet-activating factor, TNF-α, mast cell tryptase and many other cytokines, which have a linkage to neutrophil influx, direct or indirectly. This is a reflex of high levels of histamine, leukotriene B4, IL-1, IL-2, IL-5 and IL-6, and TNF-α in blister fluids (Endo, 1992; Galli et al., 1991, 1993; Grando et al, 1989; Katayama et al., 1984; Schmidt et al., 1996[a, b]). Also, pathogenic anti-murine BP180 antibodies are responsible for BP skin lesions in mice mast cells-deficient reconstituted with neutrophils, IL-8 or TNF-α (Chen et al., 2001).

Summing up, all presented studies provide evidence that subepidermal blistering also depends on mast cells. Mast cells, through degranulation, play a crucial role recruiting neutrophils to the target tissue, following the complement activation that represents an essential player in the inflammatory cascade, which leads to blister formation in bullous pemphigoid.

### b. Accumulation of Neutrophils Interference: Step 2.2.

Neutrophils were found to be essential for dermal-epidermal detachment in the *in vitro* cryosection model of BP (Sitaru et al., 2002; Shimanovich et al., 2004). Cryosection models were used for incubation with BP serum, then it was possible to observe that complement and peripheral blood leukocytes, neutrophils, line up along the basal membrane, thereafter, the dermal-epidermal cohesion breaks down. Experiments that were performed in animal models show that, in an early phase, the neutrophil infiltration depends on the complement activation, which leads to mast cells degranulation (Chen et al., 2001; Liu et al., 1995). Mice depleted of circulating neutrophils no longer were susceptible to the pathogenic effects of anti-mBP180 IgG. Also, C5-deficient mice, resistant to the pathogenic activity of anti-mBP180 IgG, could become susceptible with intradermal administration of a neutrophil chemoattractant – IL-8 and C5a. Such results prove that neutrophils recruited to the skin via C5a-dependet pathway play a crucial role in subepidermal blister formation in experimental BP (Liu et al., 1997). Springer postulated that, when neutrophils chemo attractants are injected into the skin, it will stimulate the accumulation of neutrophils increasing the recruitment of additional neutrophils (Springer, 1994). The amplification stage of neutrophils recruitment could be induced through the liberation of several proteases, for instance gelatinase B and neutrophil elastase, which in turn are detected in lesional skin and blister fluid of BP patients (Liu et al., 1998, 2000[a, b]). Neutrophil elastase (NE), an enzyme secreted by the initial wave of activated neutrophils, was proven to be able to cleave mBP180 within the immunodominant domain NC16A, which will generate a 12 kDa digestion product that is chemotactic for neutrophils. The 12 kDa fragment – called p561 – is chemotactic for neutrophils both *in vitro* and *in vivo*. So NE might be the major protease responsible for BP180 degradation (Lin et al., 2012).

It was also demonstrated that β2 integrins were absolutely critical in subepidermal blistering experimental BP (Liu et al., 2006).

It was further proved that the disease severity has a direct correlation between neutrophilic infiltration and the degree of subepidermal blistering. The neutrophil accumulation has a required threshold for clinical blistering. For instance, a 30% reduction in neutrophil influx results in the inhibition of subepidermal blisters (Liu et al., 1997).

Yamatomo et al., challenged the absolute requirement of leukocytes for blister formation of BP. They showed that anti-hamster type XVII collagen IgG and complement could start dermal-epidermal junction separation in the absence of inflammatory cells (Yamatomo et al., 2002). And so, this finding could initiate another branch in this research.

### c. Accumulation of Eosinophils Interference: Step 2.3.

It was proven that proteolytic enzymes of eosinophils play an important role during the initial stages of blister formation in BP (Dubertret et al., 1980; Varigos et al., 1982).

The infiltration of eosinophils in the developed bullous cavity leads to the direct adhesion to basal cells and the release of their granulate content towards these target cells, suggesting that eosinophils amplify the formation of DEJ separation, in BP lesions (Iryo et al., 1992).

In about 50% of BP patients, levels of eosinophils in the peripheral blood were higher, revealing a clinical condition named peripheral eosinophilia (Bushkell and Jordon, 1983) and 70% had high levels of serum IgE (Arbesman et al., 1974).

A very recent study accessed the activity of eosinophils in BP patients and found out that blood, skin and blister demonstrated a high eosinophil activity. These eosinophils also released IL-6, IL-8 and IL-1α in the blister fluid. The major outcome of this study was the increased rate of apoptosis in the cultivated BP eosinophils. All of this demonstrates that there is an apoptosis activity of eosinophils in the peripheral blood, skin and blister fluids (Engmann et al., 2016).

Messingham et al. utilized clinical samples to prove the relationship between IgG autoantibodies and eosinophilia in BP. Samples collected from fourty-eight untreated patients diagnosed with BP revealed that peripheral eosinophil count correlates strongly with IgE autoantibodies directed against BP180. Expression of FcεRI – the high IgE receptor – by circulating and lesional eosinophils from BP patients provides a new perspective for mechanisms of action for IgE in BP. This suggests that expression of FcεRI may contribute to eosinophil degranulation in BP lesions (Messingham et al., 2011, 2014[b]). Besides, a recent study demonstrated that FcγR variations affects the susceptibility to BP, decreasing the reactive oxygen species (ROS) that neutrophils release, which translates in a reduced granulocyte responsiveness (Recke et al., 2015).

Elevated levels of IL-5 and eotaxin in blister fluid of patients with bullous pemphigoid was also observed, which could demonstrate that eotaxin level correlates with the number of lesional eosinophils and that the high level of IL-5 could indicate an association between these cytokines and eosinophil accumulation in BP. IL-5 is related to blister formation and eosinophilia in BP and this can be due to the fact that T cells and eosinophils can produce this cytokine and, therefore, be the major sources of IL-5 on blister fluid. The IL-5 promotes eosinophil growth and activation and eotaxin causes eosinophil migration (D'Auria et al., 1998; Endo et al., 1992; Shrikhande et al., 2000; Wakugawa et al., 2000). Moreover, IL-5 and eotaxin seem to be the main responsible for the increasing of the inflammatory response and to contribute to the influx of granulocytes that, through the release of proteinases or cytotoxic agents, which includes eosinophil major basic protein (MBP) and eosinophil cationic protein (ECP), will ultimately lead to the epidermis and dermis separation, at lamina lucida level of the BMZ (Czech et al, 1993).

In accordance with what was written above, eotaxin specific receptor – CCR3 – has been reported to be highly expressed on eosinophils, basophils, and Th2 cells in BP (Frezzolini et al., 2002).

Th2 cells essentially release IL-4, IL-5, IL-6 and IL-10. Through an ELISA assay, Schmidt et al., demonstrated, in 1996 that, levels of IL-4, IL-6 and IL-10 were increased in patient's blisters compared to suction blister from healthy controls. This finding seems to be important, since IL-4 is responsible for stimulation of B lymphocytes. On the other hand, the high level of IL-10 seems to be important to a well-regulated immunological system in lesion formation in BP, since this cytokine is known to have a depressant effect on cytokine production of Th1 cells and also suppresses the functions of other $CD4^+$ cell subtypes, so this may represent a natural effort against excessive tissue inflammation (Schmidt et al., 1996[b]). This is what also happens in PV, where 87.5% of patients with active disease demonstrated high levels of IL-10, whilst the blister fluid from 12.5% patients in remission demonstrated low levels of IL-10. It was hypothesized that IL-10, in both PV and BP, is produced as a local phenomenon (Bhol et al., 2000). A study using peripheral blood from pemphigus and pemphigoid patients demonstrated that IL-10 are found in lower levels in pemphigus patients whereas no statiscally significance was found

between pemphigoid patients. In pemphigus cases this decrease wasn't associated with disease severity but only with the required dosage of steroid therapy, since the decrease of B10 cells are caused by therapy (Kabuto et al., 2016). So, it is possible to conclude that IL-10 is at high levels in blister fluids but not so much in the peripheral blood of BP and PV patients.

In 1996, a study found out that in blister fluid of BP patients there were high levels of IL-8, about 10 times higher than in suction blisters of control subjects, and knowing that IL-8 has a chemotactic effect on both T cells and neutrophils, this high levels can lead to infiltration of these cells (Schmidt et al., 1996[a]). Again, in 2000, a study indicates that BP-associated autoantibodies to BP180 ectodomain, somehow, trigger a signal that leads to expression and secretion of IL-6 and IL-8 from human keratinocytes (Schmidt et al., 2000).

Narbutt et al. conducted a study to evaluate the serum levels of IL-6 in patients with active disease (n=19) and in remission (n=24). They found that in both cases the levels of this IL was increased comparing with healthy controls (n=19) (Narbutt et al., 2008).

Another cytokine was studied for its involvement in Th2 lymphocytes and eosinophils recruitment during bullous pemphigoid – IL-16. This cytokine was already described as an essential chemotactic cytokine for a variety of $CD4^+$ immune cells. It is an immunomodulatory cytokine contributing to the regulatory process of $CD4^+$ cells recruitment and activation at sites of inflammation during Th2-mediated immune disorders, such as autoimmune diseases (Cruikshank et al., 2000; Mathy et al., 2000). Seem that IL-16 is mainly expressed by keratinocytes and $CD4^+$ lymphocytes in lesional skin of BP patients. These findings suggest that IL-16 serum levels could reflect disease activity in these patients. The significant production and release of IL-16 helps to maintain and amplify immunological process underlying blister formation (Frezzolini et al., 2004).

Also, Th17 cells and the released cytokine IL-17 may have a role in the pathogenesis of BP, contributing for the neutrophil infiltration and eosinophil recruitment, which cannot be explained through the presence of autoantibodies. There is evidence that IL-17 is implicated as pathogenic factor in other autoimmune diseases, such as systemic lupus erythematous, atopic asthma, etc. IL-17 is important for the initiation and maintenance of many autoimmune reactions. It is involved in the production of important pathogenic BP factors, such as pro-inflammatory cytokines, matrix metalloproteinases and recruitment of neutrophils and eosinophils. It also induces Th2 cells activation (Toosi and Bystryn, 2010).

In 2011, lesional IL-17 was evaluated quantitatively suggesting that, compared with pemphigus; BP shows more Th17 cell-related inflammation and less Treg-related regulation. These results could be explained by the difference in pathogenesis between pemphigus and BP, being inflammation more crucial for the blister formation in BP than in pemphigus (Arakawa et al., 2011).

Fang et al. also demonstrated how some inflammasome components and IL-18 can interfere with disease activity too. They concluded that mRNA levels of inflammasome components were up-regulated, when compared with healthy controls. Also, these components interfere with autoantibody titers for BP180-NC16A. The IL-18 levels were increased in serum, blister fluid and lesional skin, and as with inflammasomes, serum IL-18 level interfere with anti-BP180-NC16A autoantibody concentrations in patients. All of the concentrations decreased dramatically after treatment (Fang et al., 2016).

Moreover, a recent study intended to investigate the microenvironment of lesional skin and serum of BP patients. The outcome demonstrated a dense deposition of periostin in the lesional dermis of BP patients. This increased concentration of periostin could stimulate $CD163^+$ tissue-associated macrophages (TAMs) to produce autoimmune-related chemokines and cytokines, such as CXCL5 and IL-36γ (Fujimura et al., 2016).

**d. Proteolytic Enzymes Events: Step 2.4.**

Many studies have been referring high levels of proteolytic enzymes in blister fluid from BP patients, such as, neutrophil elastase (NE), cathepsin G (Grando et al., 1989), collagenase (Oikarinen et al., 1983; Welgus et al., 1986), plasminogen activators (Gissler et al., 1992; Jensen et al., 1988), matrix metalloproteinase-2 (MMP-2, gelatinase A), MMP-9 (gelatinase B) and MMP-13 (Niimi et al., 2006; Ståhle-Bäckdahl et al., 1994), playing a mechanistic role by degrading connective tissue components of the dermis and the DEJ.

NE is strongly expressed by eosinophils and is thought to proteolytically degrade extracellular matrix proteins as well as the extracellular domain on BP180 (Verraes et al., 2001). MMP-9 is secreted inactive and it's activated extracellularly, by plasmin, which is generated from plasminogen through tissue plasminogen activator (tPA) and/or urokinase plasminogen activator (uPA) and other MMPs. This leads to the conclusion that the major function of plasmin early in BP development is activating MMP-9 (Liu et al., 2005). Schmidt et al., went further and demonstrated the elevated expression and release of plasminogen activator (PA) from normal human keratinocytes upon stimulation with antibodies to human BP180. Since keratinocytes secret PA, they might play an active role in blister formation (Schmidt et al., 2004). In addition to plasmin, the MC-specific serine protease MCP-4 (chymase) also could activate MMP-9 (Coussens et al., 1999; Liu et al., 2005). After MMP-9 activation, it proteolycatilly inactivates α1-proteinase inhibitor – inhibitor of NE – which allows the free NE activity (Liu et al., 2000[a]).

Very recently, a study was performed as an attempt to find a connection between BP outcome and C-X-C motif chemokine 19 (CXCL10) levels, since this chemokine has been associated with several autoimmune diseases. Sixteen skin biopsy speciments, sera from one hundred and forteen patients and blister fluid from twenty-three were used to investigate CXCL10 expression and function. Blister fluids and sera revealed high levels of CXCL10. Also, CXCL10 serum levels seem to increase around day 60, but only in patients that relapsed within the first year of treatment. An important fact is that neutrophils and monocytes from BP patients, but not lymphocytes, reacted to CXCL10 by increasing MMP-9 secretion. It is important to note that high levels of CXCL19, BP biomarker, play into neutrophil and monocyte-associated MMP-9 release and disease relapse (Riani et al., 2016).

All these findings together indicate that proteolytic enzymes released from inflammatory cells damage the BMZ directly, which causes DEJ separation.

**e. Direct Mechanisms: An Alternative Step**

There is evidence indicating that cell-matrix adhesion could be disrupted by autoantibody binding itself, through the antibody's variable regions, independently of their fragment crystallisable (Fc) portions. The mere binding of the antibody to the BP180 ectodomain might trigger blister formation, through the impairment of these molecules, by competing

with the natural ligand and by blocking the key binding sites along the BP180 antigen (Ghohestani et al., 2001).

Macropinocytic pathway also could induce the reduction in the adhesive strength and a loss of expression of BP180, by internalization. The BP180 internalization induced by BP-IgG occurs rapidly, within < 30 minutes. However, it was proven to be insufficient to induce blister formation. Since blistering requires various inflammatory responses at the cell-extracellular matrix zone, it was speculated that it could first occur the deterioration by BP180 internalization. Blister formation in BP requires both BP-IgG – induced BP180 internalization and FcR-independent and FcR-dependent immune responses (Hiroyasu et al., 2013).

A study was conducted using various FcγR-deficient mouse strains which demonstrated that tissue destruction seems to be mediated by FcγRIV, FcγRIII and FcγRIIB, whereas FcγRI was not pivotal. For instance, pharmacological inhibition of FcγRIV and depletion of granulocytes seems to abolish skin blisters. This also reinforces that Fc receptor (FcR) – independent mechanisms may contribute to pathogenesis (Schulze et al., 2014).

The activation of pro-inflammatory cytokines by autoantibodies might constitute another mechanism of blister induction, by activation of intracellular signaling pathways, thus resulting in hemidesmosomal disassembly. So, in 2000, Schmidt et al. demonstrated that BP IgG induces the release of IL-6 and IL-8 from human keratinocytes, directly modulating these cytokines (Schmidt et al., 2000).

### 2.5. Concluding Remarks

Although over the years of research animal models have generated discussion topics due to their unpredictability, these models revealed to be very important in the study of the pathomechanism of BP. These models provide tools that help us to better understand the mechanisms underlying the disease.

As perspective, urges the need to design novel and more specific therapeutic strategies to counteract the chronic morbidity and mortality. This new design may arise from further investigation of the inflammatory cascade that is generated in the disease.

The novel therapies may involve the use of monoclonal antibodies able to modulate the immune response. Moreover, induction of immunological tolerance could also offer a valid alternative, avoiding the use of corticosteroids, for example.

In short, the **Figure 8** summarizes the main pathophysiological mechanisms that happen in BP, such as the bind of IgG to BP180, the cleavage of BP180 by NE and the bind of BP230 to IgE. Most of these events culminate with mast cell degranulation, which in turn will lead to blister formation.

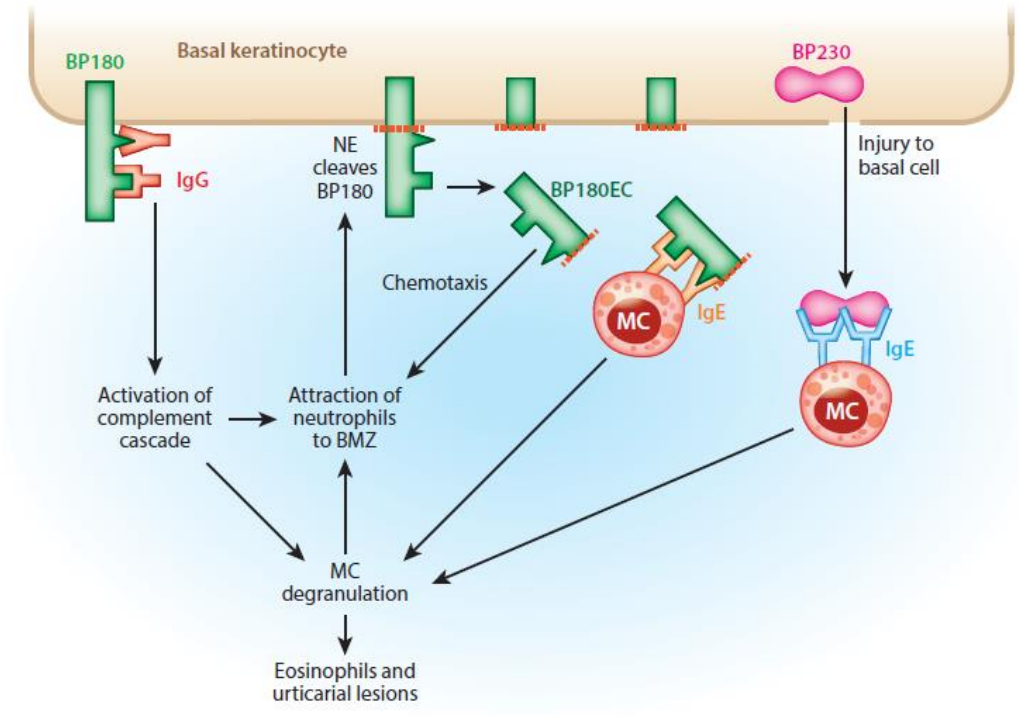


**Figure 8 – Important pathophysiological pathways in BP summarized (from Hammers and Stanley, 2016).**

In general, autoantibodies need to interact with factors of the innate immune system, which includes complement system and inflammatory cells, in order to induce blisters (Sitaru and Zillikens, 2005).

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# Chapter III

## Mechanisms of the disease and Molecular Basis: Pemphigus Vulgaris

Pemphigus vulgaris is a mucocutaneous blistering disease characterized by IgG autoantibodies against the stratified squamous epithelium (Kalantari-Dehaghi et al., 2013). However, in a small sample of patients with both PV and PF, desmogleins are not recognized by IgG, instead IgA against Dsg1 and Dsg3 is present, in these cases it is necessary further investigation to determine the impact of the IgA antibodies on phenotype and if this could interfere with therapeutic regimen (Mentink et al., 2007).
A mice model with a deletion of epidermal-specific desmocollin 3 (Dsc3) demonstrated that Dsc3 also constitutes an antigen in PV. Moreover, the incubation of patient IgG with human keratinocytes led to the loss of intercellular adhesion. This suggests that PV has a pattern that could result from autoimmunity of Dsg3, Dsc3 or both. Also, this event was later observed in other forms of pemphigus (Mao et al., 2010; Rafei et al., 2011).
The classic forms of PV are divided in two subtypes, according to antibody profile. These are mucosal dominant type of PV that only have anti-Dsg3 IgG autoantibodies and the mucocutaneous type of PV that have both anti-Dsg3 and anti-Dsg1 IgG autoantibodies (Ding et al., 1997). The role of Dsg4, a new isoform, has been investigated in disease development. Dsg4 was reported in thirty (77%) patients of a cohort with 39 PV and PF patients' sera. These results suggest that Dsg4 might have other role besides adhesion (Nagasaka et al., 2004).
In the past decades, studies of autoimmune response in PV have been updated, which led to a major outcome: analyzing levels of antibodies to Dsg3 by enzyme linked immunosorbent assay (ELISA). This represents an important milestone and a diagnostic criterion of PV (Amagai and Stanley, 2012). Desmogleins are transmembrane glycoproteins of desmosomes that confer cell-to-cell adhesion within the epidermis (Hammers and Stanley, 2016), represented in **Figure 9**.

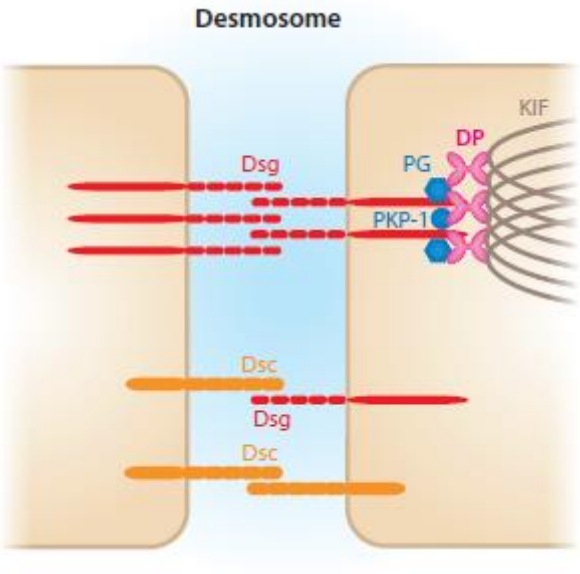


**Figure 9 – A scheme with the intercellular keratinocyte desmosome. The desmogleins, that are targets of the pemphigus antibodies, are responsible for the mediation the cell-to-cell adhesion. The connection is also mediated by desmocollins. Abbreviations: Dsc, desmocollin; Dsg, desmoglein; DP, desmoplakin; KIF, keratin intermediate filament; PG, plakoglobin; PKP-1, plakophilin 1 (adapted from Hammers and Stanley, 2016).**

The desmosomes are complex structures, disc shaped, also known as a macula adherens, with is specialized for cell-to-cell adhesion. The intercellular space is wide, with about 30 nm. Desmosomes are transmembrane proteins that bridge the space between adjacent epithelial cells by way of homophilic binding of their extracellular domains to other desmosomal cadherins on the adjacent cell. The desmosome extracellular domain is called the extracellular core domain (ECD), and is bisected by an electron-dense midline where the desmoglein and desmocollin proteins bind to each other (Amagai, 2003; Junqueira and Carneiro, 2013; Waschke, 2008). It is known that desmosomes are dynamic structures with an exclusive plasticity, required in some particular vertebrate tissues needing strength, extensibility and elasticity, such as epithelium (Celentano and Cirillo, 2016).

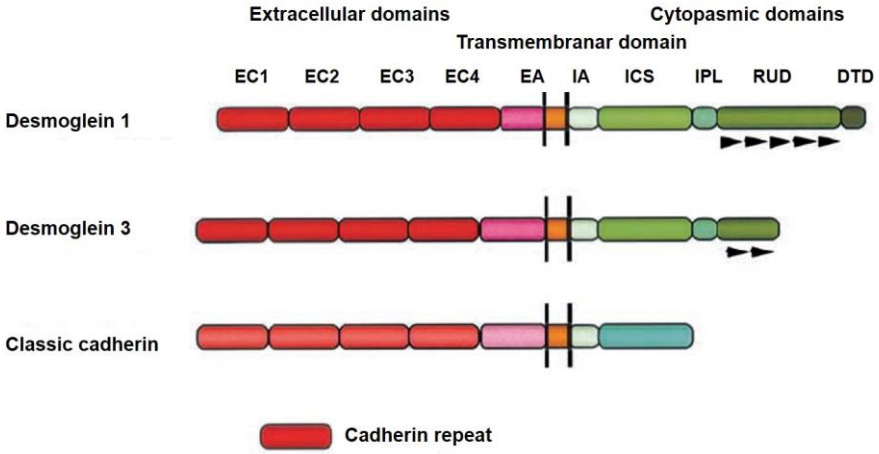


**Figure 10 – Molecular structure of desmoglein. The extracellular (EC) region of each cadherin member contains four cadherin repeats, which have calcium-binding motifs, with about 110 amino-acid residues. Desmogleins have their own unique sequences with 291 residues. The boxes with the same color have similar amino acid sequences. DTD: desmoglein-specific terminal domain; EA, EC anchor domain; IA, intrecellular achor domain; IPL, intracellular proline-rich linker (adapted from Amagai, 2003).**

Through the use of keratinocyte cDNA expression library with PV serum, it was possible to determine that the amino acid sequence of the cell surface 130 kd glycoprotein, PV antigen (PVA) had a significant homology with members of the cadherin family of $Ca^{2+}$ dependent cell adhesion molecules, desmoglein, which is now called Dsg 3 (Amagai et al., 1991).

In 1992, Amagai et al. demonstrated that PVA has five ectodomains – EC1 to EC5 – and localized the immunogenic domains. These findings defined that the major pathogenic epitope was the amino-terminal extracellular domains, a crucial region for hemophilic adhesion of cadherins (Amagai et al., 1992), as seen in **Figure 10**. Moreover, these findings suggest a direct inhibition of adhesive interaction of Dsg as an initial molecular event of blister formation in pemphigus (Tsunoda et al., 2003). To prove the crucial role of pemphigus antigens for keratinocyte adhesion, a genetically engineered mice with Dsg3 gene deletion were created, which demonstrated the critical role of Dsg3 for adhesion (Koch et al., 1997). This kind of discovery allowed the development of ELISA to diagnose

PV patients more effectively. A study confirmed that out of fourty-nine PV patients' sera, fourty-six were positive in the Dsg3 ELISA. Moreover, this ELISA also provides a correlation of disease activity with serum antibody levels (Ishii et al., 1997).
Also, a very recent study found out that pemphigus, PV and PF, patients had altered levels of prolactin (total and free) and dehydroepiandrosterone sulfate (DHEAS), sex hormones. A cohort with fifty-two newly diagnosed pemphigus patients from Theran, Iran, was accessed and investigators reported that pemphigus' patients had higher levels of prolactin and lower of DHEAS. Pemphigus patients with a more severe form of the disease had increased levels of serum total prolaction (Yousefi et al., 2016). These recent findings could be another open door to pemphigus pathogenesis studies, about the role of these sex hormones in the course of disease.

### 3.1. Autoantibodies Interference

The pathogenicity of circulating IgG antibodies from patients with active pemphigus has been confirmed by passive transfer experiments in mice (Anhalt et al., 1982).
Nineteen PV patients' sera was characterized and all of them showed high titers of IgG autoantibodies, predominantly IgG4 (Ding et al., 1997). A case report of a male newborn diagnosed with PV represented a case in which the autoimmune response was studied at molecular level, being possible to demonstrate that neonate's antibody to Dsg3 mainly belonged to IgG4 class. In other words, it was once again illustrated the blister inducing capacity of anti-Dsg3 antibodies of IgG4 class (Parlowsky et al., 2003). Another study with PV patients determined the enrichment of IgG4 in these patients. The Dsg-specific antibodies contain about 7.1% of total IgG4 in PV patients, which represent eight-fold enrichment of IgG4, when compared with age-matched controls. Also, IgG4 depletion in PV sera was responsible for the pathogenicity reduction in a keratinocyte dissociation assay (Funakoshi et al., 2012).
The disease can be induced in cultured human keratinocytes, with recombinant monovalent single-chain variable-region fragments (scFvs) cloned from PV patients, proving that antibodies directly mediate acantholysis (Payne et al., 2005).
The transmission of PV during pregnancy, to neonatal babies, is a clear evidence of passive transfer experiment in humans (Kardos et al., 2009). For instance, Dsg3 antibodies per se can induce the skin blistering in the newborn. This is the result of a different distribution pattern of the Dsg1 and Dsg3 (Avalos-Díaz, et al., 2000).

#### 3.1.1. In: Cell Adhesion

As previously referred, some pemphigus antibodies directly interfere with cell adhesion.
Since the discovery of autoantibodies interfering with desmosomal adhesion molecules it has been investigated the autoantibodies direct interference with desmoglein binding, in a so called "Direct steric hindrance model" (Bystryn and Grando, 2006; Waschke, 2008).
It was proved, in 2008, that Dsg3 autoantibodies in PV directly inhibit Dsg3 *trans* interaction. In this experiment it's possible to confirm that PV-IgG directly interfere with hemophilic Dsg3. PV-IgG reduced binding activity of Dsg in ~60%. Also, PV-IgG caused keratinocyte dissociation as well as loss of Dsg3 *trans* interaction (Heupel et al., 2008).

Moreover, studies comparing polyclonal PV patients IgG and monoclonal Dsg3 antibodies, showed that polyclonal PV IgG causes extensive clustering and endocytosis of keratinocyte cell surface Dsg3, while, pathogenic mouse monoclonal antibodies compromise cell-to-cell adhesion strength without causing these alterations in Dsg3 trafficking (Saito et al., 2012).
Further studies were made in order to find a direct inhibition of adhesion and epitopes mapping. A study demonstrated that 77.5% of the dominant epitopes bound by pemphigus sera mapped to the N-terminal extracellular domain of Dsg3 which, by analogy to classic cadherins, is crucial for adhesion (Sekiguchi et al, 2001).
Moreover, findings suggest that the disease activity is more correlated with the level of immunoreactivity against the mature form of Dsg by ELISA than against the premature one. Another interesting finding suggests that much of the immunoreactivity in pemphigus sera target very restricted regions of Dsg that are masked by the prosequence, therefore, unmasked by proteolytic cleavage of prosequence. This leads us to the pathogenic hot spot on Dsg (Yokouchi et al., 2009). Besides the importance of interruption of *trans* adhesion of Dsg, it was also reported that patients could harbor pathogenic antibodies that also target the *cis* adhesive interface within the amino-terminal extracellular domain – ectodomain (Di Zenzo et al., 2012). This kind of adhesion of desmoglein could be essential in the strengthening of cell-to-cell adhesion, by clustering of the desmoglein in desmosome. Another important fact is that pemphigus antibody binding to desmogleins is essentially dependent on their normal, calcium-stabilized conformation (Kamiya et al., 2013).

### 3.1.2. In: Desmoglein Clustering/Internalization

Other model that could explain the loss of cell-to-cell adhesion is the desmoglein nonassembly depletion hypothesis. This hypothesis tells us that maybe the loss of cell adhesion is due to the ability of multivalent pemphigus anti-desmoglein antibodies to crosslink and, possibly, cluster desmogleins. The crosslinking results in internalization of the nonjunctional Dsg and prevents the newly synthesized desmoglein from being incorporated into the desmosome. Ultimately, this lead to depletion of the desmosome of desmoglein and thus won't be able to provide adhesion (Oktarina et al., 2011). PV patients have been reported with clustering of Dsg3 by anti-Dsg3 autoantibodies (van der Wier et al., 2014). Several studies of cell cultures, such as human squamous cell carcinoma cell line and primary human keratinocytes, were made and revealed similar results of Dsg3 clustering and depletion in desmosomes (Aoyama and Kitajima, 1999; Jennings et al., 2011; Stahley et al, 2014).
Moreover, Mao et al. demonstrated, using primary human keratinocytes, that even monovalent human PV anti-Dsg mAbs reproduce the effects of polyclonal PV IgG on Dsg3, meaning that these antibodies can deplete Dsg3 incorporation in newly formed desmosomes (Mao et al., 2009).
Jennings et al. proved that is possible to prevent the process of Dsg3 disassembly expressing exogenous Dsg3 with an adenovirus delivery. This could prevent the Dsg3 loss in the desmosome and also prevent acantholysis (Jennings et al., 2011).

### 3.1.3. In: Cell Signaling & Loss of Cell-to-Cell Adhesion

Besides the steric hindrance model that could explain the underlying molecular mechanisms for the loss of intercellular adhesion, there is a speculation of the plakoglobin (PG) involvement, which causes interference of desmosomal cadherin-bound antibody with intracellular events. To prove this hypothesis, Caldelari *et al.* used knockout embryos ($PG^{-/-}$) and control mice ($PG^{+/+}$), and only $PG^{+/+}$ keratinocytes responded with keratin retraction and loss of adhesion. Also, in these cells, PV IgG binding severely affected the linear distribution of plakoglobin at the plasma membrane (Caldelari et al., 2001).

Later, more studies showed that PG suppresses c-Myc expression and, on the other hand, PV antibodies trigger c-Myc upregulation by depletion of plakoglobin and Dsg3. Once c-Myc is increased, this will cause a cell proliferation and a weak cell-to-cell adhesion. It is possible to inhibit the PV antibodies to cause acantholysis in mice, through pharmacological inhibition of c-Myc (Williamson et al., 2006, 2007).

Another cascade of signaling that has been exhaustively studied in pemphigus mechanisms is the p38MAPK signaling pathway, showed in **Figure 11**. Both, HSP27 and p38MAPK has been subject of studies where it is demonstrated that both are phosphorylated upon incubation of human keratinocyte cell cultures with PV IgG and, also, both are linked to Dsg3 internalization (Berkowitz et al., 2005; Rubenstein and Diaz, 2006). It is possible to inhibit p38MAPK, using a tandem peptide (TP). Spindle et al. proved that this peptide sequence was capable of inhibit both autoantibody-induced p38MAPK activation and its association with Dsg3, abolished p38MAPK-induced keratin filament retraction, and promoted desmosomal Dsg3 oligomerization (Spindler et al., 2013). More authors reported that that p38MAPK and HSP27 inhibitors prevent PV blistering disease *in vivo* (Berkowitz et al., 2006).

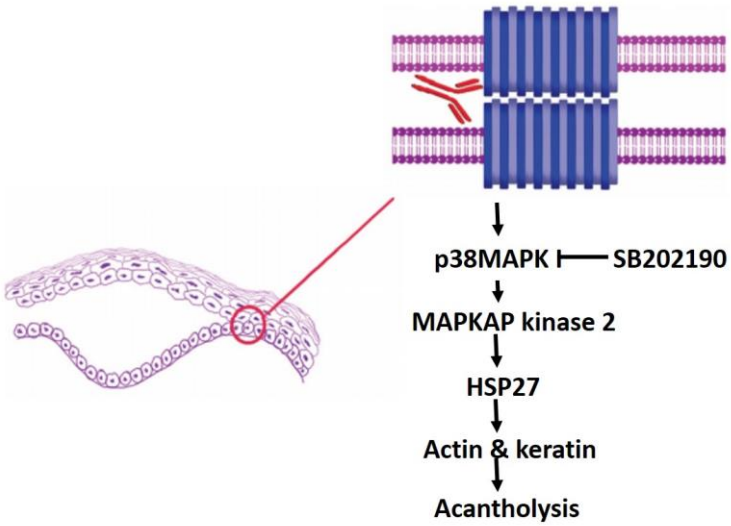


**Figure 11 – Proposed model that can explain the PV acantholysis by phosphorylation of p38MAPK. The autoantibody binds to Dsg3 and induces the phosphorylation, which is associated with keratin filament retraction, actin cytoskeletal remodelling and loss of cell-to-cell adhesion. These events will provoque the blistering. The keratinocyte p38MAPK inhibition could block these events in tissue cuylture and blistering *in vivo* (adapted from Berkowitz et al., 2006).**

This signaling cascade was already seen in PV patients' skin (Berkowitz et al., 2008).
However, is not fully clarified if p38MAPK activation is a primary event causing acantholysis or if it's secondary to initial loss of cell adhesion. Studies with monoclonal PV antibodies (opposing the polyclonal PV IgG) demonstrated that p38MAPK isn't required for loss of intercellular adhesion but it could potentiate endocytosis of Dsg3 and blistering (Mao et al., 2011). It is noteworthy that monoclonal pathogenic antibodies rely on steric hindrance to cause loss of intercellular adhesion but don't rely on p38MAPK signaling cascade. On the other hand, polyclonal PV IgG could cause Dsg3 clustering and endocytosis through p38MAPK-dependent way (Saito et al, 2012).
The epidermal growth factor receptor (EGFR) also plays an important role in blistering. In 2013, Bektas *el al.* showed that EGFR could be activated in primary human keratinocytes. The EGFR inhibition were able to lock PV IgG-triggered Dsg3 endocytosis, keratin intermediate filament retraction, and loss of cell-to-cell adhesion *in vitro*, which prevent blister formation in the passive transfer mouse model in pemphigus. So, there is a cross-talk between Dsg3 and EGFR that is regulated by p38MAPK. Pharmacological inhibition of EGFR signaling could represent a treatment to prevent blister formation (Bektas et al., 2013).

### 3.1.4. Desmoglein Compensation Explanation

Sera samples from twenty-four patients with mucosal dominant PV and twenty with mucocutaneous PV were obtained during clinically active disease and the concentrations of Dsg1 and Dsg3 antibodies were measured, by ELISA. This study revealed that all patients with mucosal dominant PV were negative against Dsg1 and positive against Dsg3, and all patients with mucocutaneous PV were positive against both Dsg1 and Dsg3.

This proves that PV patients show a dual status of serum antibodies (Amagai et la., 1999). Autoantibody profiling and determination of normal tissue distributions of Dsg3 and Dsg1 gives us the tools needed to explain the different histological sites of blister formation in mucosal PV and mucocutaneous PV – desmoglein compensation model (Amagai et al., 1996; Mahoney et al., 1999), as exemplified in **Figure 12**.

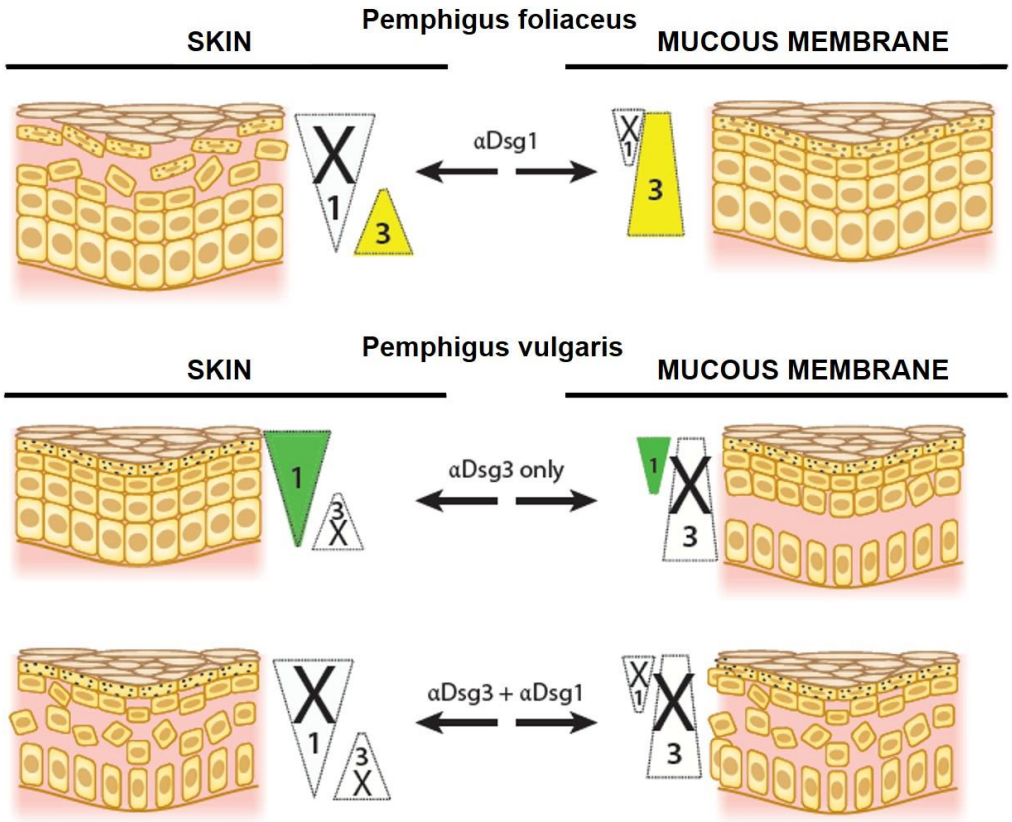


**Figure 12 - Desmoglein compensation model, for PV and PF. The triangles show the usual localization of Dsg1 (green) and Dsg3 (yellow) in the epidermis and mucous membrane. The triangle width refers to the relative amount of Dsg present at each cell level. The loss of color in a triangle represents loss of function of that particular Dsg due to the presence of αDsg1 and αDsg3. When the Dsg1 and Dsg3 function has been inactivated and the other Dsg is not present to compensate the loss, blistering occurs. Abbreviations: αDsg1, anti-Dsg1 antibodies; αDsg3, anti-Dsg3 antibodies; Dsg, desmoglein (adapted from Hammers and Stanley, 2016).**

What this model tells us is that anti-Dsg1 or anti-Dsg3 antibodies only inactivate their specific Dsg. Meaning that, if both Dsg1 and Dsg3 are present, in any level of the epidermis, and only one is inactivated, the other will compensate and provide adhesion. But if only one desmoglein is present at a particular level of epidermis and it is also inactivated, then acantholysis occur (Hammers and Stanley, 2016; Waschke, 2008). Using an animal model, it was possible to see that in Dsg3 knockout neonatal mice, skin blisters do not occur. This happens because Dsg1 is present in the epidermis to compensate for the lack of Dsg3. The opposite happens when anti-Dsg1 antibodies are transferred to these mice, which resulted in a severe PV-like blistering (Mahoney et al.,

1999). So, once again, it was possible to demonstrate that the loss of both Dsg3 and Dsg1 induce cutaneous blisters, like those seen in PV patients.

### 3.2. T Cells Interference

Several studies strongly support an association between certain human leukocyte antigen (HLA) class II alleles and PV susceptibility. Also, these studies demonstrate the prevalence of PV in certain ethnic groups (e.g., Ashkenazi Jews). All this suggest that PV patients must have HLA class II molecules that are able to present Dsg3 peptides to T cells (Ahmed et al., 1990; Sinha et al., 1988; Wucherpfennig et al., 1995; Yan et al., 2012). So, how do these molecules present Dsg peptides to T cells? It was ascertained that the major T cell population was stimulated when antigenic peptides of the extracellular domain of Dsg were presented to T cells are the $CD4^+$ memory T cells. When stimulated, these cells secreet Th2-like cytokines. Also, it was demonstrated that the antigenic response of Dsg3-specific T cells was restricted to HLA class II alleles DRB1*1401 and DRB1*0402. These results hypothesized that both alleles are restricting elements for T cell response to Dsg3 in patients with PV (Lin et al., 1997).

A study carried out by Veldman et al., in 2003, concluded that, Dsg3 reactive Th2 cells appeared in all of their PV patients, in similar frequencies, whereas, autoreactive Th1 cells exceeded the Th2 cells concentrations in chronic active PV. Healthy carriers of HLA class II alleles, DRB1*0402 and DQB1*0503, exhibited exclusively Dsg3-reactive Th1 cell responses, while healthy carriers of other HLA class II alleles did not. Also, IgG1 and IgG4 against Dsg3 were directly related to ratio of Dsg3-reactive Th1/Th2 cells. All this suggests that appearance of Dsg3-reactive Th2 cells is restricted to patients with PV (Veldman et al., 2003).

An analysis to the peptides that these Dsg3-specific T cells recognize set out that mostly come from the amino-terminal extracellular domain of Dsg3. Also, they can share anchor residues in positions 1, 4 and 6, and that position 4 is positively charged and is crucial for binding to the negatively charged surface (p4 pocket) of DRB1*0402 (Veldman et al., 2004[a]). Meanwhile, a HLA class II tetramer-based detection system was developed with DRB1*0402 tetramers loaded with immunodominant peptides of Dsg3, which has a great potential providing a new approach monitoring *ex vivo* T cells active autoimmune response against Dsg3 in PV patients (Veldman et al., 2006).

To strengthen the fact that Dsg3-specific $CD4^+$ T cell responds to HLA-DRB1*0403, it was showed in a humanized HLA-DRB1*0402 transgenic mouse model that HLA-DRB1*0402 restricted T cell recognition of human Dsg3 epitopes could lead to pathogenic IgG antibodies (that recognize both amino and COOH-terminal epitopes of Dsg3 ectodomain) induction, which could cause the loss of epidermal adhesion. These results showed that $CD4^+$ T cells recognize immunodominant Dsg3 epitopes, *in vivo*. Once again we are facing another potential therapeutic target; the Dsg3-reactive $CD4^+$ T cells (Eming et al., 2014). Hennerici et al. conducted a study that aimed to analyse the cytokines derived from antigen-presenting cells (APC) and the relation with $CD4^+$ T cell, and also the relation with autoantibodies response in pemphigus. Samples were taken from peripheral blood of fifteen PV patients and three PF patients. It was observed that plasma concentrations of APC-derived immunomodulatory cytokine IL-27 were highly increased, and IL-27 is strongly related to Dsg-specific IgG autoantibodies. Moreover, Th 17 cells and T follicular

helper (Tfh) cells had high concentrations. Plasma concetrations of IL-21also were increased, which is produced by Th 17 and Tfh cells. This suggests that both IL-27 and IL-21 have a role in pemphigus pathogenesis (Hennerici et al., 2016) and, knowing that cytokines are important in mediating T cell function (Giordano and Sinha, 2012) it could be important to further investigate the role, and characterization, of IL-27 and IL-21 (Hennerici et al., 2016).

Moreover, the $CD4^+CD25^+$ regulatory T cells (Treg) also have been suggested to play a role in the maintenance of the peripheral tolerance to Dsg3 in mice, suppressing the $CD4^+$ effector T cells, Th1 (Veldman et al., 2004[b]; Yokoyama et al., 2011).

It is important to note that a couple of studies found a central mechanism of T cell tolerance induction within the thymus described for Dsg3 (Mouquet et al., 2008[a]; Wada et al., 2011). Although, the exact contribution for health and disease are not very clear and deserve more investigation, in the future.

A recent study also demonstrated the role of $CD163^+$ tissue-associated macrophages with the dense deposition of periostin in lesional dermis of PV patients (Fujimura et al., 2016). Findings suggested that periostin interacts with its own functional integrin receptor molecules in order to induce production of proinflammatory cytokines from keratinocytes to accelerate Th2 type immune responses in allergen induced skin inflammation (Masuoka et al., 2012).

### 3.3. B Cells Interference

Over time, researchers tried to fulfil the need to understand the function of human pemphigus antibodies, cloning the anti-Dsg B cell repertoire from patients with PV in order to generate and analyse mAbs. To further demonstrate the importance of the cloning of mAbs, sera from a patient with active PV were tested with antibody phage display. It was isolated human anti-Dsg mAbs as single-chain variable-region fragments (scFvs), which demonstrated binding to Dsg3, Dsg1, or both Dsg3 and Dsg1. Also, these cloned antibodies demonstrated that idiotypes on pemphigus antibodies could be shared between patients (Payne et al., 2005).

To better understand how antibodies cause pathogenicity, sera from three patients with PV were analysed, by antibody phage display (APD). The heavy chain complementary-determining region 3 (H-CDR3) of most of the pathogenic mAbs, but not non-pathogenic, shared an amino acid consensus sequence, D/E-X-X-X-W (D/E being acidic amino acids and W is tryptophan), where tryptophan seem to have a critical position in Dsg-mediated adhesion (Yamagami et al., 2010).

Using human-mouse heterohybridomas, anti-Dsg hybridomas (IgM and IgG) it was also possible to evaluate that about 90% were specific for Dsg1 and Dsg3, which indicates extensive cross-reaction. This study reveals that variable heavy (VH) gene has more impact in pathogenicity and Dsg binding than the variable light (VL) (Qian et al., 2007).

EBV-transformed B cells were also used to clone Dsg3-specific IgG antibodies from PV patients. Some of them revealed to be pathogenic in a keratinocyte culture dissociation assay. They also made an epitope mapping where it is possible to be notice that pathogenic antibodies disrupted Dsg3 *cis* interactions, and not the *trans* (Di Zenzo et al., 2012).

A genetic analysis was also performed in peripheral blood of four PV patients to clone Dsg3-specific B cells. The results showed that the variable heavy gene VH1-46 has the most important usage, being favoured in anti-Dsg3 B cells, even though these cells use various VH genes for autoantibodies production (Cho et al., 2014).
There is a case report that suggests that localized PV without detectable antibodies can lead to systemic PV. Two patients, after suffering from systemic PV, developed long-lasting localized PV, with no serum anti-Dsg autoantibodies; however, the antibodies against Dsg3 were detectable in the systemic stage. The biopsy of localized lesions revealed suprabasal acantholysis. So, it is important to stay alert to the possibility that localized PV could appear after amelioration of the systemic stage and the decrease of pathogenic antibodies. The localized PV responds well to topical immunosuppressive, with no need to increase the immunosuppressors dose, such as prednisolone (PSL) (Yoshifuku et al., 2016).

### 3.3.1. B Cells: When They Persist

Pemphigus patients, with the active disease, revealed clonal expansions of B cells (Hammers et al, 2015; Mouquet et al., 2008[b]). Once the complete remission (with anti-CD20 antibody), the CDR3 lengths seems to be normalized, which indicates a normal B cell repertoire. However, a patient with relapse after remission reveals new clonal expansion, and in a patient in incomplete remission the original clonal expansion maintains. These findings could suggest that certain specific clones of anti-Dsg B cells may be eliminated by adequate treatment. Although, remains the doubt of whether the same pathological clones persist or even if new clonal lines could appear in the circulation of patients that relapse (Mouquet et al., 2008[b]). So, Hammers et al. characterized the autoimmune B-cell response in patients with active and relapsing disease, cloning the anti-Dsg3 IgG+ B cells. The results demonstrated that nontolerant anti-Dsg3 B cell lineage persist in patients that relapse. Even in patients in periods of complete remission, off therapy, and also in patients in multiple courses of rituximab, an antibody that ablates $CD20^+$ B cells. In two patients, in long and complete remission off therapy, it was not possible to detect anti-Dsg3 $IgG^+$ B cell clones anymore (Hammers et al., 2015). These data is in line with previous findings about anti-Dsg3 B cell receptors not being found in remissive patients (Colliou et al., 2013).
These findings indicate that it's possible that some clones are hidden, and could reappear to cause a relapse. Maybe the loss of B cells ability to tolerate Dsg3 is time-limited, which will allow clones of anti-Dsg B cells to escape tolerance and to be able to proliferate in the periphery. In the contrary, it is believed that this defect in tolerance for newly B cells should not persist and that means that if therapy is capable of destroying all nontolerant anti-Dsg3 B cell clones, the newly formed won't also be able to escape tolerance when B cell repertoire is re-established. It was also believed that if all anti-Dsg3 B cells were not eliminated, in patients, with therapy, they could proliferate and, by differentiation in short-term plasmocytes, increase anti-Dsg antibody production and cause the relapse of the disease (Hammers and Stanley, 2016). Hammers and Stanley did an analogy between cancer and pemphigus, meaning that if we could get rid of all the abnormal cells, we can cure the disease (2016).

### 3.4. Other Mechanisms Misleading Cell-to-Cell Adhesion

Not only the autoantibodies or mutations are associated with the disruption of desmosomal adhesion. Lately other discoveries were made regarding the toxins influence in this kind of disruption in the epidermis.

Recently, flotillin has been connected with cell-to-cell adhesion, as reviewed by Bodin et al. (2014). Flotillin, 1 and 2, are ubiquitous proteins associated with lipid microdomains, also called membrane rafts. These proteins are present in many cellular compartments, which include the plasma membrane and endosomes. They have been implicated in cellular signaling and membrane trafficking processes – endocytosis and endosomal trafficking (Banning et al., 2014[a]). Moreover, Völllner et al. demonstrated that the flotillins depletion in human keratinocytes cause an impairment of desmosomal adhesion and a decrease of Dsg3 expression. The loss of flotillin seems to induce a mislocalization of the Dsg3 pattern, which is very similar to the localization of Dsg3 upon treatment with PV autoantibodies. However, Dsg1 showed a decrease in flotillin-2 knockdown cells, whereas flotillin-1 knockdown cells demonstrated a high variable expression (Völllner et al., 2016). Both flotillin-2 and -1 lose their expression almost entirely in knockout mice (Banning et al., 2014[b]), while the knockdown cell line exhibits loss of only one specific flotillin (Völllner et al., 2016).

The study of apoptosis could also be a tool to better understand the variety and complexity of pathophysiologic events (Grando et al., 2009; Schmidt and Waschke, 2009). Using TUNEL technique (Terminal deoxynucleotidyl transferase dUTP nick end labeling), tissue from fifteen PV patients were analyzed. Positive results were observed in basal layer cells in 14/15 (93,3%), while in 13/15 (86,7%) of the cases granular layers that formed the blister room were noted and in 12/15 (80%) of the cases the presence of positive acantholyc cell was confirmed (Cuevas-Gonzalez et al., 2016). TUNEL assay is a tool used to detect apoptosis, since during this event, nuclear endonuclease-digested genomic DNA is fragmented into oligonucleases (180 – 200 bp), which allows DNA fragments to be identified by the catalytic addition of 16-dUTP to the free ends by terminal deoxynucleotidyl tranferase (TdT) (Gavrieli et al., 1992; Sharma et al., 2016[a]).

Another study analysed the tissue from twenty-five PV patients with the TUNEL assay. It was reported that 76% of the cases revealed acatholytic cells (Deyhimi and Tavakoli, 2013).

The implication of apoptosis in the pathophysiology of pemphigus vulgaris could, in the future, lead to the development of apoptotic blockers as therapeutics.

However, a review article from Bektas et al., suggest that apoptosis might not be necessary to occur blister induction. In other words, it could be the activation of proapoptotic proteins (caspase cysteine proteinases) that sensitize cells for the acantholytic effects of pemphigus IgG (Bektas et al., 2010). Although caspases are usually implicated in cell apoptosis, they also have other nonapoptotic biologic functions (Schwerk and Schulze-Osthoff, 2003) and one of them is precisely regulate the desmosome assembly and disassembly. Caspases have been demonstrated to have the ability to cause cleavage of desmosome proteins, so this could be important to Dsg physiological behaviour in the keratinocytes (Bektas et al., 2010).

As for BP and PF, so in PV the complement activation seems to be quite important. Components of both the classical and the alternative pathway are found in PV lesions (Panelius and Meri, 2015). The components C1q, C4, C3 of the complement were already

found in PV (Jordon et al., 1985). Not only these components were found but also the MAC-neoantigen have been found, both in PV and PF (Kawana et al., 1989). By IIF assay of skin biopsies it is possible to see deposition of C3 and IgG in keratinocytes (Sitaru et al., 2007).

### 3.5. Concluding Remarks

As I aforementioned, direct and indirect mechanisms contribute to loss of desmoglein-mediated adhesion, which leads to pemphigus acantholysis. We can ascertain that acantholysis initiates with cellular signaling pathways and not by direct inhibition of Dsg binding. Also, some mechanisms causing acantholysis are found to be exclusive in PV, so they don't contribute for acantholysis in PF, despite the similarity of both diseases.

As I reported, there are many possibilities regarding the mechanisms that could possible trigger blistering in pemphigus diseases but still there are only few certainties about how exactly this happens.

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# Chapter IV

## Mechanisms of the disease and Molecular Basis: Pemphigus Foliaceus

Pemphigus Foliaceus is the most superficial version of pemphigus. This variant shows cutaneous lesions and virtually no involvement of mucous membranes associated with subcorneal cleavage and autoantibodies against Dsg 1 (Mihai and Sitaru, 2007), the antigen with ~ 160 kDa (Koulu et al., 1984), in which PF antibodies seem to bind to a calcium sensitive epitope of Dsg1 (Eyre and Stanley, 1987). Contrary to what happens in PV, where patients have Dsg3 and Dsg1-specific antibodies, in PF it seems that only Dsg 1 is reported, due to desmoglein compensation, as it is possible to see in **Figure 13** (Hammers and Stanley, 2016). The sera of a PF patient were used to isolate anti-Dsg1 mAbs as single-chain variable fragments (scFvs). The scFvs proved to cause blistering both in mice and human epidermis models, which demonstrates that a single monoclonal antibody is able to corrupt Dsg1 function and, accordingly, cause the disease. The scFvs isolated showed affinity to bound to conformational epitopes in the amino terminal of Dsg1. This study also proved the restriction of the heavy-chain gene usage of all anti-Dsg1 clones (Ishii et al., 2008).

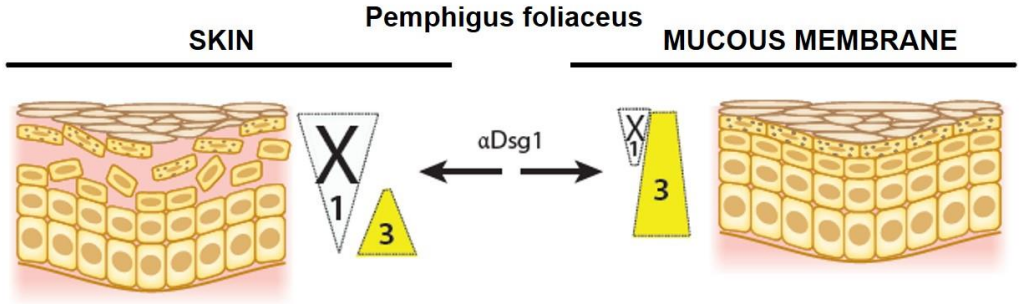


**Figure 13 – The desmoglein compensation theory in PF. The yellow triangles represent the usual localization of Dsg3 in the epidermis (skin) and mucous membrane. The width of the triangles indicates the relative amount of desmoglein present at each cell level. Loss of color in the triangle represents loss of function of that particular Dsg due the presence of αDsg1, in this case. Abbreviations: αDsg1, anti-Dsg1 antibodies (adapted from Hammers and Stanley, 2016).**

### 4.1. Blister Formation Mechanisms

Experiments in animal models reported a clear pathogenicity of circulating IgG antibodies. More specifically IgG4 subclass seems to be predominant in PF patients (Rock et al., 1989).

Moreover, PF can be induced both in mice and in human skin organ culture. These experiments demonstrate that antibodies can directly induce acantholysis (Rock et al., 1990). As represented in **Figure 15** and mentioned above, when, in a particular level of epidermis, only one desmoglein is present that will easily lead to acantholysis. So, assuming that PF is mainly characterized by Dsg 1 autoantibodies, when this desmoglein is inactivated, superficial blisters will occur. However, passive transfer experiments in mice models with PF anti-Dsg1 antiobodies have shown that the deep epidermis can express Dsg3 too, and this will help to maintain adhesion at that epidermis level (Wu et al., 2000).

PF pathophysiology is much similar to described above for PV, so direct inhibition of cell adhesion also happens in PF patients. Epitope maping studies with PF patient's points out that most dominant epitopes mapped the amino-terminal ectodomains of Dsg1, which are crucial to cell-to-cell adhesion (Sekiguchi et al., 2001).
A case report also suggests that, not only desmogleins have an impact in PF pathogenesis, but also desmocollins (Dsc) have an important role. This case reported IgG reactivity specifically against desmocollins. This strong reactivity was confirmed by ELISA, repeated several times, with serum samples taken before and after treatment. All these taken together give a strong suggestion of the pathogenic Dsc1. This case gives us an alternative direction in pemphigus' diagnosis, in cases without detectable anti-Dsg autoantibodies (Geller et al., 2016).
Again, as detailed above for PV, the Dsg disassembly depletion hypothesis is also crucial to PF pathophysiogy. The concept of this hypothesis, even when applied to PF, is the same as I explained for PV, the loss of cell-to-cell adhesion is due to anti-Dsg antibodies to crosslink and agglomerate desmogleins (Oktarina et al., 2011).
The p38MAPK signalling cascade can also be linked to Dsg1 internalization, and its pharmacological inhibition can block blister formation in PF, facts that have been proven by passive transfer mouse models (Berkowitz et al., 2008[a]). It was ascertained that the hypothesis reported in acantholysis in pemphigus patients seems to work independently (Hammers and Stanley, 2016).

### 4.2. Different Forms Of PF

Pemphigus Foliaceus comprise different clinical forms, sporadic and edemic being two of them (Joly and Litrowski, 2011). The sporadic form is rare, with only 20% to 30% of pemphigus cases with and incidence of one case per million inhabitants per year, in Europe and USA (Joly and Litrowski, 2011). Among the additional clinical forms of PF, *Fogo Selvagem* is also well-known and shares clinical, histo and immunopathological features and is classified as a subtype of PF (Eyre and Stanley, 1988), which was described by Cazenave in 1844 and firstly reported in 1903 in Brazil (Sampaio et al., 1994). *Fogo selvagem* fits the endemic form of PF (Aoki et al., 2011). The endemic pemphigus foliaceus (EPF) has a unique epidemiologic profile, including endemic areas, familial cases, with no differences in gender distribution (Hans-Filho et al., 1999).
The cause of FS seems to be related to environmental factors (e.g., molecular mimicry due to infections transmitted by insects) (Otten et al., 2014). It mainly occurs in rural areas of Brazil and, usually, vanishes after urbanization of the endemic areas (Culton et al., 2008). It is also possible to find the endemic PF in other countries such as America – Colombia (Abrèu-Velez et al., 2003), Venezuela, Peru, Ecuador and Paraguay - and also in Northern Africa (Tunisia) (Morini et al., 1993). This EPF form seems to have different patterns, Columbia pemphigus and Tunisian pemphigus (Joly and Litrowski, 2011).
*Fogo selvagem* is a superficial cutaneous form of pemphigus and histologic results reveal subcorneal acantholysis. In these cases, patients seem to lack mucosal involvement, contrary to what happens in PV. Serological results demonstrate pathogenic anti-Dsg1 autoantibodies in patient's serum (Amagai et al., 1995; Rock et al. 1990; Roscoe et al., 1985).

Moreover, FS mainly focuses in peasants dedicated to outdoor activities, and also has a greater incidence with geographic and familial clustering (Diaz et al., 1989; Hans-Filho et al., 1996).

### 4.2.1. Fogo Selvagem

Brazilian investigators from the University of Brasilia evaluated and treated many patients with FS, over the last four decades (Ribeiro et al., 2005; Rocha-Alvarez et al., 2007).
Culton et al. have been systematically collecting clinical and serological data from FS patients from the ameridian reservation of Limão Verde, as well as normal individuals that live in reservation and around it and they found that 55% of ameridian' normal individuals have anti-Dsg1 antibodies (Warren et al., 2000), which can be related to physical proximity to this reservation (Culton et al., 2008). *Fogo Selvagem* reveals remarkable and unique features like geographic and temporal clustering of cases and the increased incidence in young adults and children. Also, this pemphigus form has a familial cluster (Moraes et al., 1997).
It has been reported that a significant number of FS patients and healthy inidviduals that live in the endemic areas demonstrated increased levels of IgM anti-Dsg1. This suggests that environmental antigenic exposure might induce FS (Diaz et al., 2008). Possibilities have been suggested to explain the persisting IgM autoantibodies in these individuals; one of them is the polyclonal activation of IgM memory B cells (Bernasconi et al., 2002; Reynaud et al., 2012). Studies have demonstrated that both IgE and IgG4 antibodies against sand fly are present in FS patients. Also, it was suggested that IgG4 reacts to both exogenous and endogenous antigens (Qian et al., 2012). If an IgE anti-evironmental antigen was developd, this could help as a useful marker to early detect individuals at risk. Moreover, when comparing IgE anti-LMJ11 (sand salivary gland antigen, explained in Triggers in PF) levels in individuals before and afetr FS onset, the pre-FS demonstrated lower levels of anti-Dsg1 IgE, meaning that LJM11 is the main target of an IgE response. FS patients, besides having IgG4 anti-Dsg1, also have IgG4 anti-JLM11 antigen from sand fly (Qian et al., 2015).
There are three theories that determine if IgG4 anti-Dsg1 and anti-LJM11 responses are associated. First, and most importante, is that the immune responses to Dsg1 and LJM11 have two IgG4 antibodies population and these two populations overlap somehow. Second, is that IgG4 responses are independet and, third one, is that IgG4 has the same response to Dsg1 and LHM11 and all IgG4 antibodies are cross-reactive (Qian et al., 2012).

## 4.3. B cell Response

The anti-Dsg B cell repertoires have been cloned from PF patients, as well as from PV patients, generating monoclonal antibodies (mAbs), using the APD (Ishii et al., 2008). In a PF patient, in whom autoantibodies against Dsg1 cause the blistering, it was possible to clone mAbs, using the APD and isolate antigen-specific mAbs. These monoclonal antibodies were directed against mature Dsg1 (matDsg1), as seen in previous studies about Dsg3 and PV patients, on the cell surface of keratinocytes and precursor Dsg1 (preDsg1) in the cytoplasm. This study shows that individuals without PF don't have B cell

tolerance to preDsg1 and the loss of tolerance to mature Dsg1 isn't due to epitope shifting of anti-preDsg1 B cells. So, people without pemphigus also have B cells that code for antibodies against preDsg1, but lack antibodies against the mature cell surface protein. This suggests that B cell tolerance in normal people is for the exposed, matDsg1 (Yamagami et al., 2009). So, when B cell loses tolerance for Dsg1 that will allow clones of anti-Dsg1 B cells to escape and prolipherate in periphery, however, this will not happen with newly formed B cells and so if therapy is efficient enough all nontolerant anti-Dsg1 B cell will be eliminated. But if not all were eliminated, the clones will then proliferate and cause a relapse of disease (Hammers and Stanley, 2016).

Also, studies confirmed that idiotypes on pemphigus antibodies could be shared across patients. Moreover these studies suggested that Dsg binding mainly select the variable heavy (VH) and not the variable light (VL) region (Ishii et al., 2008).

Besides the cloning studies, the heterohybridoma was also used to investigate PF aetiology, more specifically, the endemic form *Fogo Selvagem*. A cohort with nine PF patients was used to collect hybridomas that secret IgM or IgG. It was reported that these anti-Dsg1 autoantibodies showed specificity toward Dsg1, so anti-Dsg1 response in FS is mainly managed by antigen (Qian et al., 2009).

Also, the cross reactivity of epitopes on LJM11 and Dsg1 IgE reacts against Dsg1. So, the chronic stimulation of LJM11 antigen and IL-10 production can promote IgG4 antibodies development, which in turn cross react to both LJM11 and Dsg1 (Qian et al., 2016).

### 4.4. T cells Response

Mechanisms of T cell tolerance were already demonstrated for Dsg1, within thymus. It was reported that Dsg1 is expressed by CD19+ CD63+ cells, so thymic expression of a tissue-specific autoantigen that could be involved in an autoimmune disease can also participate in the tolerance induction of Dsg1-specific T cells (Mouquet et al., 2008).

### 4.5. Other Mechanisms Leading to Blister Formation

The mechanism that leads to neutrophilic infiltrates and pustule formation in PF is still not clear, however, hypothesis have been made, like the possibility that complement activation could stimulate neutrophil recruitment (Matsuo et al., 2001; Panelius and Meri, 2015). Also, the IL-18 role in neutrophil chemotactic promotion was also suggested as a mechanism in pemphigus variants (O'Toole et al., 2000).

Very recently, it was found that levels of vascular endothelial growth factor (VEGF) were increased in PF patients with erythroderma (Miyamoto et al., 2016). Erythroderma is a clinical skin condition present in patients with other cutaneous disorders, and this sharing disorders may be due actions of signal protein, for instance, VEGF. This protein is released by keratinocytes after skin damage to intervene in repair responses (Creamer et al., 1996; Elias et al., 2008). This result suggests that maybe the blood vessel endothelium in PF pathogenesis. It should be noted that it was also found an association between anti-Dsg1 and VEGF which could indicate that VEGF has a suppressive response upregulation, during erythrodermic phase in PF patients (Miyamoto et al., 2016).

Recently, a case of a twenty-four years old female patient was reported with an atypical PF form, with neutrophilic pustules. Only a few cases were reported (Méndez-Flores et al., 2016).

### 4.6. Concluding Remarks

All these findings abou FS patients' points to salivary gland protein components from sand fly as FS' trigger. Suggesting that, non-infectious antigens can induce autoantibodies to harm the host. This could represent the only pemphigus form with the environment that directly influences the mechanisms that trigger this disease. It is also important to note that we are talking about the most endemic form, with a specific incidence. The mean age of onset is also affected, as well as the environment in which those who suffer most from this disease live.

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# Chapter V

## Diagnosis and Symptoms

## 5.1. Clinical Evaluation

Physical assessment is the first milestone concerning pemphigus tracking. Examination of the skin, mucous membranes and nails of the patients should be astutely done. Along with these observations, patients should be questioned about symptoms that might suggest an extraoral mucosal involvement. Also, there should be a careful review about patient's medical history and medications, since it is necessary to discriminate whether idiopathic or drug-induced pemphigus is present (Celentano and Cirillo, 2016).

The Tzanck smear can provide rapid cytologic information. Tzanck smear is a simple tool and cheap ancillary used for diagnosis. In ideal conditions, this test is performed on a fresh blister, with less than 24 hours old, and the material is gently scraped from the vesicles' base, blister or pustule, onto a slide. It's allowed to air dry and stain with different dyes (Giemsa, toluidine blue and methylene blue) (Durdu et al., 2008; Kelly and Shimoni, 2009).

In 2016, Zhou et al. conducted a study to evaluate the diagnostic value of indirect immunofluorescence microscopy (IIF), ELISA and Tzanck smear test for pemphigus diagnosis. They studied a cohort with thirty-three patients with pemphigus and sixty-one controls. The sensitivities and specificities of all the three techniques are summarized in **Table 1**.

**Table 1 - Sensitivity and specificity value for each assay (from Zhou et al., 2016).**

| Technique | Sensitivity (%) | Specificity (%) |
|---|---|---|
| **Tzanck smear** | 96.7 | 60.0 |
| **IIF** | 84.8 | 91.8 |
| **ELISA** | 84.8 | 96.7 |

These results are in line with results of some previous studies (Durdu et al., 2008; Zagorodniuk et al., 2005). This study revealed the same sensitivity value for IIF and ELISA; however the specificity value of ELISA was higher.

Although the three techniques can be used in pemphigus diagnosis, the three can also present some drawbacks that probably could affect the diagnosis. For instance, IIF has an overall sensitivity dependent on the type of substrate used (Delmonte et al., 2001). In ELISAs' case, the technique only detects circulating antibodies but no cytological message presented (Anand et al., 2011).

Tzanck smear test relies on acantholysis process. This information coupled with the one aforementioned, offers a more immediate answer, which makes it a more rapid and valuable cytological diagnostic technique (Zhou et al., 2016).

### 5.1.1. Bullous Pemphigoid Diagnosis

Early on in the disease, it can present an acute or subacute clinical picture and mostly be associated with intense pruritus (Feliciani et al., 2009). Clinically, BP patients demonstrate generalized inflammation of blistering skin. The blisters usually are tense, and heal without scarring formation. They generally arise on the distal extremities, the trunk and intertriginous areas. Moreover, oral and ocular mucosa involvement is not common, and, even if present, doesn't have any clinical significance (Otten et al., 2014).

Elderly people have a priority regarding BP, meaning that this disease should be suspected in this age group. Patients' exhibit generalized itchy erythematous papules urticarial and/or skin blistering. These blisters are subepidermal and have inflammoatory cell infiltrates, eosinophils or neutrophils.

BP diagnosis is confirmed by IIF, which has to reveal a linear deposition of IgG and C3 at DEJ of patients' perilesional skin. Furthermore, it is necessary to see if circulating IgG autoantibodies bind to epidermal side of 1 M NaCl-split skin. Adding to this, ELISA is used to measure circulating autoantibodies against BP180 and BP230, and could be used to monitor and guide decisions in diseases' course (Otten et al., 2014). The diagnosis of drug-induced bullhous pemphigoid (DIBP) is almost the same as for classical BP (Ruocco and Sacerdoti, 1991).

A study with fourteen BP patients investigated the correlation between clinical severity and BP180 ELISA indices. The commercially available BP180 ELISA kit were better following disease activity when compared with IIF, so this system unveiled a great tool to evaluate disease activity and determine the effectiveness of the treatment in BP patients (Tsuji-Abe et al., 2005). Another recent study provided an initial "proof of concept" in the use of Reflectance confocal microscopy (RCM) as an accurate, rapid and non-invasive method for BP diagnosis (Samhaber et al., 2016). This technique is an *in vivo* skin imaging that at a cellular level helps us to visualize horizontal planes from the epidermis to upper dermis (Ahlgrimm-Siess et al., 2008; Rajadhyaksha et al., 1999). This kind of technique helps physicians examine many lesions very quickly and due to the non-invasive characteristic of the technique, it is possible to follow up the natural evolution of any lesion (Grönemeyer et al., 2014).

### a. Scoring Systems: Quantifying Disease Extension

It was developed for BP a system that measures disease activity in order to standardize clinical assesements, the BP Disease Area Index (BPDAI). This system has scores for skin and mucous membrane activity and the damage score are included to help physicians separate lesions that do and do not represent active disease. Additional importance is given to arms and legs and less to face and scalp. Even though it's rare to see mucous membrane involvement in BP, their evaluation is contained in the questionnaire with the purpose of comparing the disease activity to extend of mucous membrane involvement in different ABD. Prutitus has a prominent place as it is the main symptom of BP, and the BPDAI measures the severity of this symptom, and only pruritus related to BP is considered in the system definition (**Figure 14**). For instance, if the patient has some disability, like dementia, that can stop him from completing a reliable visual analog scale rating, the degree of pruritus is inferred based on excoriations alone. This itch evaluation is not intended to be combined with the objective part of BPDAI (**Figure 15**) (Murrell et al., 2012).

**BPDAI PRURITUS COMPONENT – VAS**

**DATE**: Year/ Month/ Day _____ /____ /___

| | | | |
|---|---|---|---|
| ☐ | Baseline | ☐ | Beginning consolidation |
| ☐ | Consolidation phase | ☐ | End of consolidation |
| ☐ | Tapering phase | ☐ | Partial remission on minimal therapy |
| ☐ | Complete remission on minimal therapy | ☐ | Partial remission off therapy |
| ☐ | Complete remission off therapy | ☐ | Flare |

**A. How severe has your itching been over the last 24 hours?**

| None | | | | | | | | | | Severe |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | | | | | | | | | | |

Score out of 10 =

**B. How severe has your itching been the past week?**

| None | | | | | | | | | | Severe |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | | | | | | | | | | |

Score out of 10 =

**C. How severe has your itching been the past month?**

| None | | | | | | | | | | Severe |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | | | | | | | | | | |

Score out of 10 =

Average (**INTENSITY SCORE FOR PAST MONTH**) = **(A + B + C) =** /30

Or

**For BP patients with impaired mental functioning:**

| | | |
|---|---|---|
| ☐ | No evidence of itch (no excoriations) | **0** |
| ☐ | Mid itch (isolated excoriations up to two body sites) | **10** |
| ☐ | Moderate itch (excoriations on 3 body sites, impairment of daily activity | **20** |
| ☐ | Severe itch (generalized excoriation, sleep impairment) | **30** |
| ☐ | **TOTAL SCORE** | /30 |

**Figure 14 – Subjective BP Disease Area Index (BPDAI) pruritus score. Visual Analog Scale (VAS) (from Murrell et al., 2012).**

| BDPAI | | | | | |
|---|---|---|---|---|---|
| **SKIN** | **ACTIVITY** | | **ACTIVITY** | | **DAMAGE** |
| **Location** | Erosion/blisters | Number of Lesions if <3 | Urticaria/Erythema /Other | Number of Lesions if <3 | Pigmentation /Other |
| | 0 Absent | | 0 Absent | | Absent 0, Present 1 |
| | **1**, 1-3 lesions, none > 1 cm diameter | | **1**, 1-3 lesions, none > 6 cm diameter | | |
| | **2**, 1-3 lesions, at least 1 > 1 cm diameter | | **2**, 1-3 lesions, at least 1 > 6 cm diameter | | |
| | **3**, >3 lesions, none > 2 cm diameter | | **3**, >3 lesions, or at least 1 > 10 cm diameter | | |
| | **5**, >3 lesions, at least 1 > 2 cm diameter | | **5**, >3 lesions, at least 1 > 25 cm diameter | | |
| | **10**, >3 lesions, at least 1 > 5 cm diameter or area | | **10**, >3 lesions, at least 1 > 50 cm diameter or area | | |
| HEAD | | | | | |
| **NECK** | | | | | |
| **CHEST** | | | | | |
| **LEFT ARM** | | | | | |
| **RIGHT ARM** | | | | | |
| **HANDS** | | | | | |
| **ABDOMEN** | | | | | |
| **GENITALS** | | | | | |
| **BACK/BUTTOCKS** | | | | | |
| **LEFT LEG** | | | | | |
| **RIGHT LEG** | | | | | |
| **FEET** | | | | | |
| **TOTAL SKIN** | **/120** | | **/120** | | |
| | | | | | |
| **MUCOSA** | Erosion/ Blisters | | | | |
| | **1**, 1 lesion | | | | |
| | **2**, 2-3 lesions | | | | |
| | **5**, >3 lesions, or 2 > 2 cm | | | | |
| | **10**, entire area | | | | |
| **EYES** | | | | | |
| **NOSE** | | | | | |
| **BUCAL MUCOSA** | | | | | |
| **HARD PALATE** | | | | | |
| **SOFT PALATE** | | | | | |
| **UPPER GINGIVA** | | | | | |
| **LOWER GINGIVA** | | | | | |
| **TONGUE** | | | | | |
| **FLOOR OF MOUTH** | | | | | |
| **LABIAL MUCOSA** | | | | | |
| **POSTERIOR PHARYNX** | | | | | |
| **ANOGENITAL** | | | | | |
| **TOTAL MUCOSA** | **/120** | | **/120** | | |

**Figure 15 – Objective BP disease area index (from Murrell et al., 2012).**

BPDAI reliability, validity, responsiveness, and minimal clinically important differences were already investigated and this system demonstrated excellent results (Wijayanti et al., 2016).

### 5.1.2. Pemphigus Vulgaris Diagnosis

Usually, PV initiates in the oral cavity and is characterized by flaccid blisters and erosions, which causes pain. These events could also cause weight loss and malnutrition. Blisters can be painful and sometimes, itchy (Amagai et al., 1999; Hertl et al., 2015; Jamora et al., 2003).

About 80% to 90% of PV patients can develop oral lesions, and among these, about 60% of the oral cases can represent the first sign of this disease (Arpita et al., 2015).

Usually lesions prevail in seborrheic areas, like chest, face, scalp and interscapular region. It is noteworthy that this disease usually doesn't have an association with major pruritus, contrary to the fingernails, which can be involved (Hertl et al., 2015).
Despite being a rare manifestation, there are references that report cases of patients with esophageal PV. Howerever, this rare occurrence could simply be a misdiagnosis or originate from the low activity of PV in this area, which in turn could represent an alarm signal to start diagnosing in a more attentive and careful manner (Khamaysi and Eliakim, 2008).
Diagnosis of patients with PV needs to be considered when in presence of persistent blisters and erosions of mucous membranes and skin. The suspicions could be strengthened by a positive Nikolsky (Celentano and Cirillo, 2016). Nikolsky sign needs to be tested applying some pressure in perilesional or normal skin in order to observe if blisters can be extended or induced in normal-appearing skin, which characterizes the pemphigus group diseases (Arndt and Feingold, 1970; Doubleday, 1987). Although it is well stablished that the mucosal involvement is the main characterization of PV, there are some case reports that introduce patients that, clinically, only show blisters and erosions in the skin without mucosal involvement. These specific cases suggest that these rare phenotypes can be due to a weak anti-Dsg3 IgG when in presence of strong anti-Dsg1 IgG autoantibodies. This suggestion is postulated using the desmoglein compensation theory (Yoshida et al., 2005).
There are two cases reported of a fifty-nine and a thirty-five year old man with PV manifestation in the penis. It has been described that PV manifests with genital lesions, but the penil involvement as a first manifestation hasn't been studied and reported enough. This represent a challenge since erosive penile lesion are usually associated with infections aetiology, so the patients were subjected to antivirals and antibiotics first, with no response, obviously (Stieger et al., 2013).

### a. Scoring Systems: Quantifying Disease Extension

Knowing that over two thirds of the clinical trials have been registered in the last decades, we reached an urge for objective measures of disease activity, in order to standardize clinical assessments among investigators. Hence, two pemphigus scoring systems were developed, which are currently in clinical use: Autoimmune Bullous Skin disorder Intensity Score (ABSIS) (Daniel et al., 2012; Pfütze et al., 2007) and Pemphigus Disease Area Index (PDAI) (Daniel et al., 2012; Rosenbach et al., 2008). ABSIS system has a maximum score of 206, in which up to 150 points reflect the percentage body surface area involvement weighted by the severity of the involvement in each specified are, up to eleven points for the extend of mucosal involvement and up to fourty-five points the patients' subjective level of discomfort associated with foods. PDAI could reach a maximum score of 250, in which up to 120 points is for the skin disease, up to 120 points is for mucosal disease and up to 10 points for the scalp involvement. Also additional thirteen points could be added for the skin damage (Payne, 2016).
Boulard et al. (International Pemphigus Study Group) defined PDAI and ABSIS cut-off values for moderate, significant and extensive disease, in which moderate included those with PDAI < 15 or ABSIS <17, significant with PDAI scores between 15 and 44 or ABSIS between 17 and 53, and finally extensive disease with PDAI ≥ 45 and ABSIS ≥ 53. It is noteworthy the authors' intention to describe disease extent instead of disease severity.

These results were based in a multicenter study of 96 consecutive incident cases of pemphigus over a 34 month period, from 31 dermatology centers in six countries (Boulard et al., 2016).
Another study, with thirty-seven patients from four dermatology centers, in Japan, were made and they proposed the following scale: PDAI scores of 0 to 8, 9 to 24 and ≥ 25, as mild, moderate and severe, respectively. The cut-off scores were based on their sensitivity and specificity, towards assessments of disease severity (Shimizu et al., 2014).
Nevertheless, Payne keeps the description of the distribution of pemphigus disease activity scores independently from the measures of disease severity, validating the cut-off values based on the statistically significant differences in median PDAI, ABSIS, Physician's Global Assessment and Dermatology Life Quality Index scores, in each subgroup (Payne, 2016).
Recently, a study intended to evaluate the three systems, PDAI, ABSIS and PVAS (another system, called Pemphigus Vulgaris Activity Score), the reliability of these indexes and the convergent validity according to anti-Dsg values. They concluded that between the three indexes, PDAI had the highest validity, making it highly recommendable to use in multicenter studies for rare diseases, such as pemphigus vulgaris. This study was made with a large number of patients with pemphigus vulgaris (Rahbar et al., 2014).

### 5.1.3. Pemphigus Foliaceus Diagnosis

Clinically speaking, PF only affects skin, while PV affects mucous membranes (mucosal PV) and later involves the skin (mucocutaneous PV) (Ding et al., 1997). In PF, the blistering lesions tend to be in seborrheic areas, such as trunk, face and scalp. The onset manifestation is usually characterized by scattered, small superficial blisters, that can easily scale into crusted erosions with puff pastry-like or cornflake appearance (Hertl et al., 2015; Otten et al., 2014).
In PF, like in PV, Nikolsky sign is also positive. However, the diagnosis needs to be confirmed by demonstration of intercellular deposition of tissue-bound and by circulating autoantibodies (with DIF and IIF, respectively). ELISA is used to assess the molecular specificity of circulating autoantibodies, using recombinant Dsg1 (Otten et al., 2014).

### 5.1.4. Laboratory Analysis

A simple standard laboratory work plan is well established regarding the pemphigus screening.
In a simple manner, this should include: lesional skin/mucosal biopsy for hematoxylin and eosin (H&E) staining; a perilesional skin/mucosal biopsy for direct immunofluorescence (DIF); and/or serum collection for indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA). The samples should be treated very careful and in a differentiated way. Samples taken for biopsies should be on an intact lesion and a punch biopsy (4mm). Some clinicians use the "stab-and-roll" technique, in which they use a size 15 scalpel blade to keep the epithelial roof in the sample. This sample should be placed immediately in a solution of 10% buffered formalin. The tissue sample used for DIF microscopy has to be taken from the perilesional area, in order to contain both epithelium and stroma. This sample should be placed in an optimal cutting temperature (OCT)

compound, frozen at -20°C and then stored at -80°, until processing (Celentano and Cirillo, 2016). But if OCT compound isn't available, the sample could be, alternatively, placed in normal saline-soaked gauze and maintained at 4°C, or in Michel's medium, which can maintain the sample at room temperature for about 6 months (Vodegel et al., 2004). Sample taken for biopsy with transmission electron microscopy (TEM) and immune-mapping is treated the same way as the routine histopathology. The sample needs to be placed, at that moment, in an electron-microscopy-specific medium and in OCT, respectively (Celentano and Cirillo, 2016).
For ELISA and IIF, assays are required 5-10 mL blood samples without anticoagulants, which are then centrifuged in order to separate plasma – contains gamma globulins – from blood cells (Celentano and Cirillo, 2016).

### 5.1.5. Histologic Recognition

**Bullous Pemphigoid Recognition** The analysis of patients' lesional skin should reveal a supedpidermal cleavage, which is associated with a dense inflammatory infiltrate, composed mainly of neutrophil and eosinophil granulocytes (Otten et al., 2014). The IIF technique allows the BP differentiation from others subepidermal autoimmune blistering diseases that also have autoantibodies that bind to the dermal side of salt-split skin (Aoki et al., 2010), **Table 2**.
Nowadays, the ELISA systems using recombinant BP180 and BP230 are largely employed to characterize the IgG autoantibodies in patients with BP (Kobayashi et al., 2002; Sitaru et al., 2007[a]; Yoshida et al., 2006).
As an alternative, BP180 and BP230 specific IgG autoantibodies can also be detected by immunoblotting, using epidermal or keratinocyte extracts (Mihai and Sitaru, 2007).

**Table 2 – Diagnostic Criteria for BP.**

| Diagnostic Means | Findings |
|---|---|
| **Clinic** | Tense blisters, erythematous plaques and pruritic papules |
| **Histology** | Sub-epidermal blister with an inflammatory infiltrate consisting mainly in eosinophils and neutrophils |
| **DIF** | Linear depositions of C3 and IgG at DEJ |
| **IIF (esophagus)** | IgG autoantibodies bind to epidermal side |
| **ELISA** | BP180 and/ or BP230-specific IgG autoantibodies |

**Pemphigus Vulgaris Recognition** There are a few histopathological characteristics in pemphigus vulgaris which facilitate the diagnosis, as summarized in **Table 3**. Those features include intraepithelial cleavage with loss of keratinocytes adhesion – acantholysis – mainly localized in the suprabasal region. The retention of these keratinocytes along the BMZ leads to an appearance that seems like a "row of tombstones". There is also an

inflammatory infiltrate in the dermis, which contains eosinophils (Celentano and Cirillo, 2016; Hertl et al., 2015).

**Table 3 – Diagnostic criteria for PV**

| Diagnostic Means | Findings |
|---|---|
| **Clinic** | Mucosal/skin blistering, inflammation, erosions |
| **Histology** | Acantholysis followed by inflammatory infiltrate, intraepithelial separation (suprabasal layer), “row thombstones” |
| **DIF** | Intraepidermal deposits of IgG and/or C3, in a “cobblestone” or “fish-net” intercellular binding pattern |
| **IIF (esophagus)** | Binding of IgG autoantibodies to epithelial cells with an intercellular pattern |
| **ELISA** | IgG autoantibodies specific for desmoglein 3 (mucosal) +/- desmoglein 1 (mucocutaneous) |

**Pemphigus Foliaceus Recognition** Regarding PF, there are histologic characteristics that support the diagnosis, such as intraepithelial acantholysis beneath the stratum corneum or within the granular layer. Also, neutrophils can be present within the blister cavity and a mixture of neutrophils and eosinophils in the superficial dermis (Celentano and Cirillo, 2016). IIF is used to detect intercellular deposits of IgG and C3, whereas serum IgG autoantibodies bind to substrates, like esophagus and human skin, with an intercellular pattern. IgG autoantibodies recognize Dsg1, and levels of Dsg1-specific autoantibodies could be correlated with the disease activity, **Table 4** (Hertl et al., 2015; Ishii et al., 1997).

**Table 4 – Diagnostic criteria for PF.**

| Diagnostic Means | Findings |
|---|---|
| **Clinic** | Fragile blisters and crusty erosions (preferentially in seborrheic areas) |
| **Histology** | Subcorneal cleavage with acantholysis |
| **DIF** | Intreepidermal deposits of IgG (and/or) C3 with an intercellular pattern |
| **IIF (esophagus)** | IgG autoantibodies bind to epithelial cells with an intercellular pattern |
| **ELISA** | Dsg1-specific IgG autoantibodies |

## 5.2. Assays

### 5.2.1. Direct Immunofluorescence Microscopy (DIF)

The aim of this technique is to detect any *in situ* accumulation of immunoreacts, which usually are Ig's and/or complement components, in the perilesional skin (Chiorean et al, 2014; Otten et al., 2014). The deposition of distinct immunoreactants in the patients' skin could be detected using a fluorochrome – labeled antibodies (e.g., specific for human IgG, IgA, IgM and C3) (Chiorean et al., 2014). Immunofluorescence microscopy remains the gold standard for pemphigus, because tissue fixed intercellular antibodies is present in about 90% of the patients. Pemphigus vulgaris and pemphigus foliaceus presents similar intercellular binding of IgG and/or C3 found in a typical "cobblestone" or "fish-net pattern" in the epidermis/epithelium (Chiorean et al., 2014).

Knowing that some countries and laboratories don't have access to many important techniques to diagnose some forms of pemphigus, such as DIF, a study was conducted to compare periodic acid-Schiff (PAS) staining and immunofluorescence patterns, with a cohort of five cases of BP and five cases of PV. PAS is a method used to detect polysaccharides, such as glycogen, and mucosubstances, such as glycoproteins, glycolipids and mucin in tissues (Warnock et al., 1988). Having knowledge of the "The Altered Glycan Theory of Autoimmunity", that suggests that each autoimmune disease should have its own glycan signature, it makes sense to use the PAS technique (Maverakis et al., 2015). Also, a 98% positive correlation between DIF and PAS was found in all samples. This indicates that, when it's not possible to perform DIF, PAS can be used in alternative, in addition to hematoxylin and eosin (H&E) staining (Abreu-Velez et al., 2016).

### 5.2.2. Indirect Immunofluorescence Microscopy (IIF)

In this assay, the aim is to detect circulating autoantibodies in patients' sera, targeting skin constituents. The patient's serum is added to normal epithelial subtract in two steps incubation. The main epithelial subtract used is the monkey esophagus, since it is well established that this type of subtract affects the sensitivity of the test. In PF and PV, the best subtracts are monkey and guinea pig esophagus, respectively. It is noteworthy that more than 80% of the patients with PV and PF have circulating autoantibodies, making this assay reliable for these autoimmune blistering disease diagnoses (Payne and Stanley, 2012).

### 5.2.3. ELISA

This assay is a sensitive and easy-to-perform test that allows the characterization of the autoantibody specificity (Mihai and Sitaru, 2007). ELISA has a high demonstrated sensitivity and specificity for BP and PV, respectively (Tampoia et al., 2012). This technique measures specific autoantibodies in the patients' serum, which is incubated in microtiter plate wells and coated with the antigen of interest. The autoantibodies that match will bind to the antigen. Then, the enzyme conjugated secondary anti-IgG antibody added will change color in proportion to the reaction. As said before, in PV and PF, the levels of anti-Dsg1 and anti-Dsg3 in serum of the patients could be related with the disease activity (Cirillo et al., 2012). This assay could be an important tool for a more

targeted treatment (Zone et al., 2009). It is significant to note that high levels of anti-Dsg1 and anti-Dsg3 could remain increased, even in phases of remission (Abasq et al., 2009). There are now ELISA kits commercially available for specific detection of serum IgG to Dsg1 and 3. The sensitivity of these tests reaches an impressive 90%, far more sensitive and specific than IIF (Payne and Stanley, 2012).

### 5.2.4. Some Additional Testing

Immunoblotting – Western Blotting – and immunoprecipitation are very sensitive assays and specific for autoantibodies detection. These techniques use recombinant autoantigens or keratinocyte extracts from healthy human skin. Moreover, protein microarrays have been used as a powerful technique for detection of a large number of autoantibodies, in autoimmune blistering diseases (Kalantari-Dehaghi et al., 2013[a]). Immunoprecipitation usually is more useful for immunoserological follow-up and as a serological confirmatory test, since it's more sensitive than immunoblotting (Celentano and Cirillo, 2016). Maybe the future lies in protein arrays, thanks to the complexity of the autoantibody profile (Celentano and Cirillo, 2016).

## 5.3. Concluding Remarks

Since the detection of tissue-bond and serum autoantibodies play an essential diagnostic role in autoimmune blistering disease, the characterization of molecular specificity of autoantibodies allows the development of reliable diagnostic algorithms. These algorithms can help clinicians in streamline this group of diseases, as we can see in **Figure 16**.

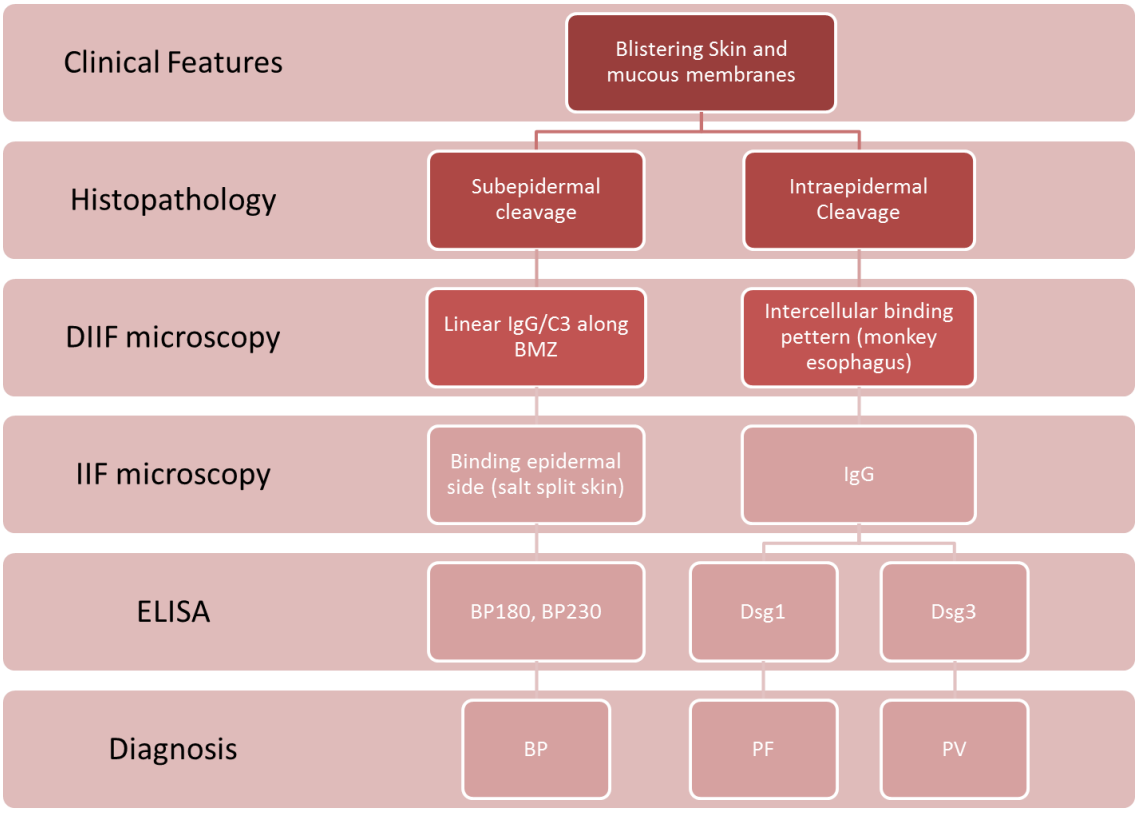


**Figure 16 – Diagnostic Algorithm for ABD.**

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# Chapter VI

## Associated Diseases - Which Induces What?

Over the years, BP has been associated with other malignancies, with several reported cases of pemphigoid associated with malignancies (PAM). Despite this, the correlation remains shrouded in controversy. The main theory that explains this association is that antibodies directed against tumor-specific antigens of malignant cells may cross-react with antigens, like the BP antigens, in the basement membrane zone, which could lead to blister formation. About forty cases of PAM were identified by Balestri et al. very recently. Of the forty cases, seven have conections with hematological malignancies and the other thirty-three with solid tumors (Balestri et al., 2016).
The incidence of malignancies in BP patients seems to be higher than in general population, ranging from 5.8% to 19%. It is also possible to observe a greater incidence in Europe (Venning and Wojnarowska, 1990; Cozzani et al., 2001), first, and the in East Asia (Ogawa et al., 1995; Peiying et al., 1993; Chang et al., 1996), despite the reduced number of studies regarding the matter.

## 6.1. Concerning Bullous Pemphigoid

### 6.1.1. BP and neurologic conditions

In the past, many reports have demonstrated an association between BP and some neurologic conditions. It has been estimated that about 36% of all BP patients had at least one neurologic disorder (Cordel et al., 2007). However, it is not well understood the underlying mechanism in this association, but it certaintly cannot be attributed to elderly age, since previous studies reported this same association in middle-aged patients. It is noteworthy that patients with more than 80 years that have neurologic diseases also have 10 times more probability to develop BP (Korman et al., 1991). It seems that BP tends to be associated with neurologic disorders that involve autoimmune mechanisms, like Parkinson and Alzheimer, since specific antibodies have been found accumulating within neurons in Alzheimer's disease, which can initiate neuronal degeneration. This degeneration, which leads to a break of tolerance, could explain the delayed BP development, after onset of neurologic symptoms (Bastuji-Garin et al., 2011). A very recent study associates BP180 autoantibodies with a more severe dementia, Alzheimer disease, suggesting a shared role of BP180 autoantigen in neuro-dermatological interactions and reinforcing the association between BP and neurodegeneratives disorders (Kokkonen et al., 2016). Some authors also reported a significant risk of cerebrovascular disease, such as Parkinson and dementia, developing subsequent to a BP diagnosis (Brick et al., 2014; Lai et al., 2016; Taghipour et al., 2010).
Stinco et al. reported that association between BP and Parkinson's disease is statistically significant, since a higher prevalence of BP in hospitalized patients with Parkinson's disease was demonstrated (Stinco et al., 2005). Rates of Parkinson's disease among BP patients have also been reported as increased in more recent studies (Bastuji-Garin et al., 2011; Cordel et al., 2007), making this information consistent. A wide study that aimed to assess BP risk in patients (n=868) with some sort of neurological disorders, such as stroke, Parkinson's disease, multiple sclerosis and epilepsy, concluded that all of them, except epilepsy, have direct association with the disease, which supports a possible causal linkage (Langan et al., 2011). It has been reported a strong association between multiple sclerosis and BP (Försti et al., 2016).

There is one Portuguese study that correlates neurologic diseases and BP.It reports that 35.1% of the patients had a stroke at some point, 37.7% had demetia and 5.2% had Parkinson's disease. All the neurologic conditions, except Parkinson's, presented an association with BP (Teixeira et al., 2014). Lai et al. also made a research to evaluate the risk that BP patients had for neurologic diseases with a pool of 23369 BP cases. Following the above, this study confirms a signification association between the BP cases and neurological diseases (Lai et al., 2016).

### 6.1.2. BP and psychiatric disorders

Some psychiatric disorders have been associated with BP, such as the bipolar and unipolar disorders, schizophrenia and personality disorders (Försti et al., 2016). The frequency of these diseases has been found to be increased; however, the association with BP is still statistically not significant (Bastuji-Garin et al., 2011). It was also demonstrated that schizophrenia has a high frequency in women with BP (Chen et al., 2011).

### 6.1.3. BP and other immune disorders

Koerber Jr, in 1978, reported the coexistent of psoriasis and bullous pemphigoid in six case reports (Koerber Jr et al., 1978) and in 1985, Grattan accesses a record of sixty two BP patients to determine if psoriasis and BP have, in fact, any association. He found that seven BP patients, 11%, had psoriasis but none of the controls had it. So it was possible to infer that the incidence of psoriasis in BP patients was significantly higher than expected, suggesting a linkage between the two diseases (Grattan, 1985). In a report case of a seventy-seven year old male patient with psoriasis later diagnosed with BP, Iskandarli et al. suggested a rule that they called "no psoriasis, no BP", which means that maybe BP can be a sign of active psoriasis, at least in this case. For instance, the occurrence of these two diseases in the patients could mean that active psoriasis induced BP, and psoriasis-induced BP can be a sign of psoriasis activity (Iskandarli et al., 2015). Many other studies have confirmed concomitant psoriasis and bullous pemphigoid in several patients, and since pathogenic relationship between psoriasis and BP is not that clear, it has been suggested that the autoimmune process responsible for BP lesions could be induced by UV light therapy, topical corticosteroids, and/or the inflammatory processes that occurs in psoriasis (Person and Rogers III, 1976; Wilczek and Sticherling, 2006). Some investigators think that maybe psoriasis vulgaris and BP occur together due to a combined action of the epidermis affected by psoriasis and the effects of antipsoriatic therapy. These combined might initiate production of autoantibodies that will attack the BMZ, in individuls prone to it. It is a fact that the majority of these reported BP cases in literature happened after antipsoriatic therapy, like anthralin, psoralens, sun exposure, PUVA therapy, among others (Ruocco et al., 2013[b]).

BP also seems to be linked to alopecia, vitiligo, and dermatitis herptiformis – Duhring, PF and PV. Although the coexistence of BP and PV or PF is very rare, there is such a case reported in the 1990's (Korman et al., 1991).

As aforementioned, eosinophils play a crucial role in BP, and are also a source of tissue factor (TF), that is an initiator of blood coagulation. Marzano et al. reported that the

coagulation cascade is activated in BP and there is a correlation between the disease severity and eosinophilia, which indicates that eosinophils also play a role in coagulation activation via TF. These events increased risk of thrombotic complications in BP (Marzano et al., 2009).

BP is also associated with diabetes mellitus. Jedlickova et al. found that the frequency of diabetes mellitus was increased in BP patients with more than eighty years, but they didn't find any statistical significance (Jedlickova et al., 2010).

Although allergy and BP have different mechanisms, growing information has been gathered that indicates an association between these two abnormalties, which suggest that these two disorders might share the same pathophysiologic mechanism (Rottem et al., 2002). However, there was established a link between allergy and autoimmunity - the antibodies IgG4 and IgE. While IgE has a crucial role in allergic diseases, IgG4 can block an inflammation induced by allergens, blocking antibodies (Gleich et al., 1982). These observations can be demonstrated taking immunotherapy as an example: levels of IgE decrease whereas IgG4 levels increase, after therapy (Golden et al., 1992). Also, these alterations in IgE and IgG4 levels could be attributed to IL-10, produced by T cells, being exposed to allergens (Akdis and Akdis, 2015). Being IgG4 antibodies the most important pathogenic autoantibodies in PV, PF/FS and BP, as aforementioned, this can represent the link between allergies and ABD.

It is also possible that the relation between IgE and IgG4 antibodies is a consequence of the sequential isotype switch from IgM through IgG4 to IgE (Jabara et al., 1993). This divergence between IgG4 and IgE may be good to patients who suffer from allergies; however, it has a negative effect in FS patients, exacerbating the IgG response which will lead to pathogenic IgG4 autoantibodies (Qian et al., 2016).

Another important fact is that, sometimes, it can happen that three or more autoimmune disorders are developed in the same patients, which is called multiple autoimmune syndromes (MAS) (Anaya et al., 2012), This syndrome was firstly described by Humbert and Dupond (1988). However, the pathogenesis of this syndrome is still unclear, so it has been proposed that the presence of one autoimmune disorder can lead to detection of other pre-existing disorders, such as vitiligo, thyroiditis, and bullous pemphigoid in the patient (Mohan and Ramesh, 2003). Sundaram et al. reported a case of an eighty-one year old male that suffered from rheumatoid arthritis, with vitiligo over the hands, later also diagnosed with BP. In this case, not only the patient had BP as he had two more diseases, which turned his condition into a treatment, diagnosis and pathomechanisms challange. Once again, it is unclear if BP was induced by the other autoimmune disorders. Further research is needed to understand this disease entity and to evaluate if BP can be prevented in these cases (Sundaram et al., 2014).

There are several reported cases of BP linkage with other autoimmune diseases, such as Sjögren's syndrome (Yamamoti et al., 1998), rheumatoid arthritis (Sundaram et al., 2014), systemic lupus erythematosus (Stoll and King Jr., 1984), multiple sclerosis (Masouyé et al., 1989; Stinco et al., 2005), , thymoma (James, 1984), psoriasis (Pašlć et al., 2002), nephrotic syndrome (Hoorn et al., 2015) and primary biliary cirrhosis (Guerra-Uribe and González-Huezo, 2016). However, there are all rare occurrences.

A potencial link between BP and amyotrophic lateral sclerosis (ALS) was also studied, and although the pathogenesis of BP and ALS is not well understood, it is possible to find some immunological aberrance (Nakane et al., 2016).

The tense, pruritic blisters that characterizes BP, sometimes can be preceded by prodromal pruritic, urticarial or eczematous eruptions. This can lead patients to develop pruritus without blisters, as a prodrome of BP (Alonso-Llamazares et al., 1998). Very recently, a seventy-five year old Japanese woman with a ten month history of widespread pruritic nodular eruptions was diagnosed with BP (Yoshimoto et al., 2016). It was determined that when BP is associated with this nodular lesions, it is called pemphigoid nodularis, which is a rare type of pemphigoid (Ross et al., 1992; Yung et al., 1981). In 1986, a case of BP after an eleven year history of autoantibody-negative prurigo nodularis was also reported (Roenigk and Dahl, 1986). These two cases suggest that pemphigoid nodularis might start with prurigo nodularis and then evolve into production of autoantibodies anti-BP180 and/or BP230.
Very recently, a case of a seventy-five year old female with lymphocytic colitis that was then diagnosed with BP was also reported (Sperl et al., 2016).
Another study seems to find a different cluster between PV and autoimmune thyroid disease (AITD), rheumatoid arthritis and type 1 diabetes, PV and systemic lupus erythematous (SLE), and AITD and rheumatoid arthritis. It could suggest that there are common genetic elements across these clinically distinct diseases. Indeed, the prevalence of AITD, rheumatoid arthritis and type 1 diabetes are increased in PV patients, when compared with the general population (Parameswaran et al., 2015).

### 6.1.4. BP and pulmonary diseases

A study from Langan et al. confirmed that BP patients had three times more probability to have pneumonia and pulmonary embolism. It is suggested that the greater risk for pneumonia in these patients maybe due to the use of high oral cortisteroids doses for BP treatment (Langan et al., 2009). In 1979, Savin also made an association between BP deaths and bronchopneumonia and pulmonary emboli (Savin, 1979).

### 6.1.5. BP and cardiovascular disorders

Since BP treatment can interfere with blood preasure and heart diseases, it is suggested that both hypertension and ischemic heart disease might play a role in BP patients. Some drugs used in hypertension are reported to induce BP (Jedlickova et al., 2010; Pietkiewicz et al., 2015).

### 6.1.6. BP and neoplasia

A recent and in-depth epidemiological study by Ong et al., from 1999 to 2011, with a cohort of 2.873.720 patients with malignant cancers, was performed. Curiously, the cohort used was not found to be at greater risk of concurrent or subsequent BP than the control cohort. However, patients with BP appear to be at greater risk in specific sub-cohorts of patients with kidney cancer, laryngeal cancer or lymphoid leukaemia. They also reported that patients with BP as a primary diagniosis had no increased risk for malignant cancers, similar to previous findings in the study (Ong et al., 2014). Very recently, a case of BP in a sixty-seven year old man diagnosed with pancreatic neoplasm, a lymphoepithelial cyst, was also reported. After the excision of the cyst, a fast improvement occurred without resorting to treatment, which suggests that maybe BP involved the cyst. It was

hypothesized that maybe the cyst triggered BP (Chadwick et al., 2016). A case report of an eighty two year old Japanese woman strengthens the bond between gastric cancer and BP. She was diagnosed with both diseases and, after surgery for gastric cancer, BP improved dramatically (Noguchi et al., 2014).

Currently there are more theories that try to explain the linkage between malignant neoplasms and BP, graphically shown in **Figure 17**. The first, and most important one, explains that there is a possibility for antibodies directed against tumor-specific antigens of that malignant cells to cross-react with BP antigens in the BMZ. This event can lead to blister formation. The mechanism behind this cross-reaction could be explained by laminin-332, an essential protein for keratinocytes attachment to epidermal basement membrane. Aware of it, laminin-332 also is expressed by some tumors, like adenocarcinomas (colon, breast, pancreas, and lung) to promote tumor growth, invasion and metastatic behavior (Endo et al., 2010; Kikuchi et al., 2011). Another theory refers the possibility that tumor cells may secrete a hormone-like substance that causes damage to the epithelial BMZ, which in turn may lead to production of anti-basement membrane antibodies. The third theory suggests that there is an intervention of an external agent, like a virus or something similar, which can be tumorigenic and induce damage to BMZ. Lastly, the appearance of a cancer could occur due to production of antibodies with capacity to cross-react with the BMZ of the skin (Dahl and Ristow, 1978; Patel et al., 2012; Venning and Wojnarowska, 1990). Again, some authors refer the possibility of a genetic predisposition, since human leukocyte antigen (HLA) DR13 has been found more commonly in patients with both BP and malignancy, when compared with patients only with BP (Venning et al., 1990). Recently, Tukaj et al. reported the pathological role of heat shock protein 90 (Hsp90), which is a cell stress-inducible molecule that participates in the development of malignancies and autoimmune blistering diseases, especially in BP. The selective Hsp90 inhibitors have been proposed for treatment of both conditions (Tukaj et al., 2015).

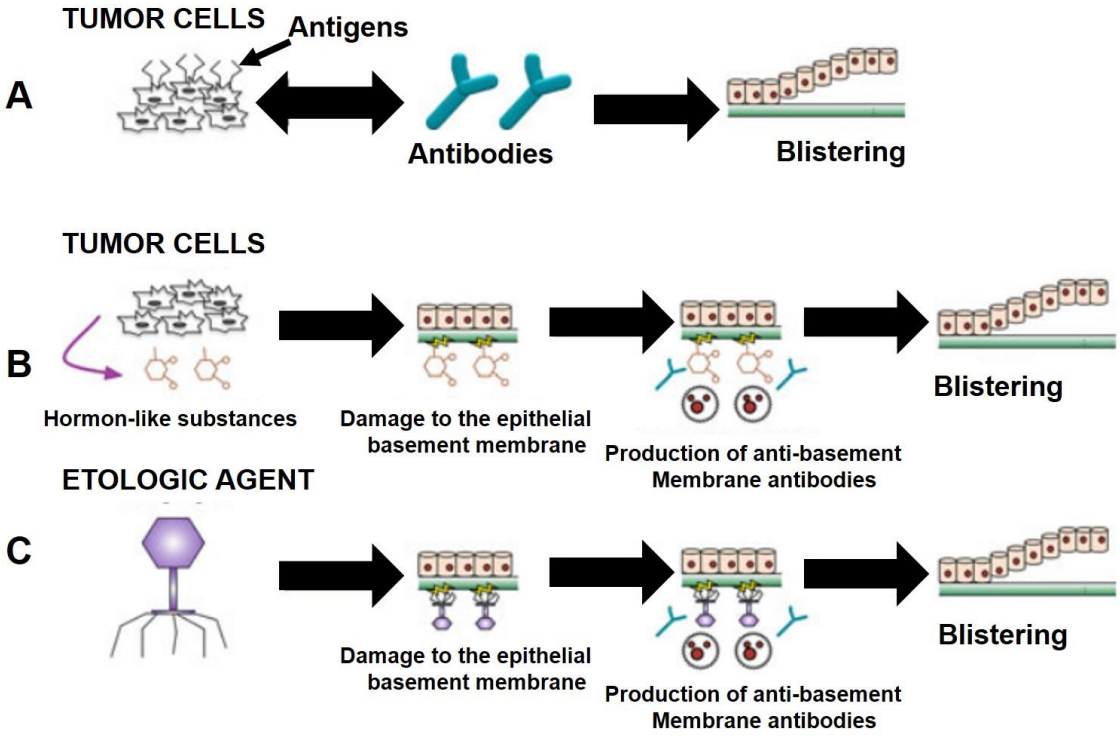


**Figure 17 - Representation of the theories that try to explain the linkage between malignant neoplasms and BP: (a) Antibodies directed against tumor-specific antigens of malignant cells cross-react with antigens (like BP antigens) in the basement membrane zone (BMZ). (b) Tumor cells secrete a hormone-like substance that could damage the epithelial BMZ. (c) Virus or other agent which is tumorigenic and at the same time induces BMZ damage (adapted from Balestri et al., 2016).**

There should be a bigger concern from physicians about PAM and an oncological screening should be carried out in early-onset pemphigoid. They should screen patients that had cancer, patients with symptoms neoplasm-related, and BP patients in immunosuppressive therapy (Balestri et al., 2016).

Ruocco et al. studied fourty-five BP patients, from 2006 to 2011, to determine the neoplasms incidence and correlate them with BP disease. They reported that 22.2% of all BP patients had some sort of neoplasm, lung cancer being the most frequent, in 6.6% of the patient, and prostate and uterus cancer being the next, with 4.4% each, as summarized in **Figure 18** (Ruocco et al., 2013).

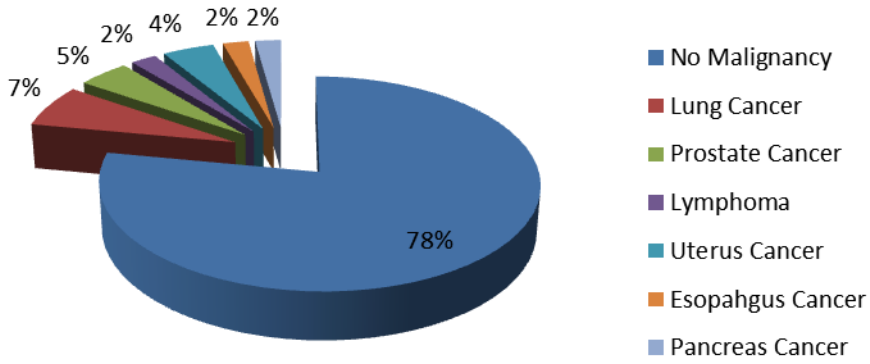


**Figure 18 – Incidence of malignancies in BP (adapted from Ruocco et al., 2013).**

There is a unique case report of an eighty-two year old diagnosed with BP that was then also diagnosed with spindle cell carcionoma of the gallbladder. BP has been associated with some neoplasias, but, for this one in specific, it was the first time, and some authors have suggested that this kind of association could make us think of possible factors of internal malignancies in BP patients (Umekoji et al., 2010).

## 6.2. Concerning Pemphigus Vulgaris

The most important comorbidities associated with pemphigus are insomnia, Cushing syndrome, adrenal insufficiency, inability to swallow due to mucositis infections with herpes and fungi. This conclusion was made upon a very wide USA cohort with 87.039.711 hospitalized patients (Hsu et al., 2016).

### 6.2.1. PV and neoplasia

Bernard et al. studied a cohort of 85 patients with thymoma, an uncommon neoplasia derived from the epithelial cells of the thymus, and found out a high relation between this disease and autoimmune diseases, in which 47/85 (55%) cases of thymoma had an autoimmune disease, and one of the patients had pemphigus. They also suggested that preexisting autoimmune diseases are not a risk factor for developing autoimmune manifestations after thymectomy. There are some theories that try to explain this association, although all of them are based in the failure of positive and negative selection of T-lymphocytes in the thymus – “escape theory”, “genetic theory” and the “AIRE (autoimmune regulator) theory” (Bernard et al., 2016).

Also, in Japan, a fifty-four year old man with thymoma was diagnosed, eight months before, with PV (Saraya et al., 2015).

### 6.2.2. PV and viruses

Knowing that viruses have a huge implication in PV patients life, a study tried to access and compare levels of IgG antibodies against herpes simplex virus type 1 and 2 (HSV1 and HSV2), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in the sera of twenty-five PV patients and twenty-seven healthy individuals. The comparison of the levels of anti-EBV, anti-CMV and anti-HSV2 IgG showed high titers of antibodies in PV patients, but not in the controls. The level on anti-HSV1 was a bit increased but the difference between PV patients and controls were not significant (Ghalayani et al., 2015). Other studies have been done over the years that could corroborate the presence of viruses in PV patients (Barzilai et al., 2007; Kalra et al., 1999; Kurata et al., 2014; Tufano et al., 1999).

Moreover, it is not clear if the presence of these viruses could induce PV in patients. The most probable is that this presence is a consequence of the disease and, hence, the treatment. Viral infections can only be casual ou be a reflection of the lack of normal epithelial barrier, or it might reveal a pathogenic link (Ruocco et al., 2014).

### 6.2.3. PV and pulmonary diseases

Despite being extremely rare, autoimmune bullous disease associated with interstitial lung disease (ILD) cases are repored for PV. Bai et al. reported one of the rare cases with this association. A fifty-three year old Chinese female with PV developed ILD after a relapse of PV. It seems that this association occurs only when autoimmune bullous diseases relapse or aren't under control (Bai et al., 2016). Also, in 1989 a case was reported of a fifty-two year old Japanese female with PV that developed ILD too (Usuba et al., 1989). ILD also developed in a seventy-three year old female, in this case with BP (Yoshioka et al., 2012).

### 6.2.4. PV and oral mucosa diseases

As aforementioned, PV affects the oral mucosa, and there is a report that describes a case of a forty-seven year old male diagnosed with PV that was later diagnosed with periodontitis (Pradeep et al., 2010). Periodontitis is known as a multifactorial disease plaque induced inflammation that involves and destroys the supporting alveolar bone, cementum and periodontal ligament (Beukers et al., 2016). This case reinforces the huge need in periodontal follow-up by dental professionals in order to prevent this kind of painful periodontal disease progression.

### 6.2.5. PV and infections

A retrospective study with one hundred and fifty five PV patients, sixty eight males and eighty seven females, from 2009 to 2011, reported a high prevalence of infections in them. Thirty-three of all patients had infection at admission (*Staphylococcus aureus* and *Escherichia coli*) and nine acquired nosocomial infections. Also, thirty seven had oral candidiasis and fifteen had localized herpes simplex. This high prevalence of infections in these patients suggests that infection is directly correlated with disease severity and presence of diabetes mellitus (Esmaili et al., 2013).

A study tried to correlate the pathogenesis of PV with *Mycobacterium tuberculosis*, screening the sera of sixty PV patients and twenty eight controls. It was found that seven

PV patients (11.7%) and none of the controls revealed presence of IgG against this bacillus. This means that PV patients seem to be more prone to exposure to *M. tuberculosis*, since maybe PV patients are sensitized against this bacterium, which means that *M. tuberculosis* contributes to PV pathogenesis (Ali et al., 2016).

### 6.2.6. PV and other diseases

Recently, it was raised an important issue that possibly links endoplasmic reticulum (ER) stress and anti-Dsg1 in PV. It was reported that the majority of the forty two oral biopsy specimens from PV patients had correlation with development of ER stress, in the course of the disease (Mihailidou et al., 2016). This stress in the ER causes functional disturbances, leading to evolutionarily conserved cell stress responses – the ufolded protein response - which wil cause cell death (Xu et al., 2005).

## 6.3. Concerning Pemphigus Foliaceus

The prevalence of metabolic syndrome (MetS) and its components were assessed form both PF and PV. A cohort with one hundred and forty seven patinets (48.9 % PF and 51.1% PV) living in the northeatern region of são Paulo, Brazil, was compared twith Brazilian casuistic samples. Although the results didn't find any significant difference between prevalence of MetS, it did find a quite high prevalence of each component of MetS, such as high blood pressure in PV male patients, and in both genders for PF; diabetes mellitus in both genders and both diseases; obesity in PV and PF female patients; and, hyperglyceridemia in both genders for both diseases again. So the components per se are more prevalent in PF and PV than in control group and this suggests that more investigation is needed to assure if this syndrome can or cannot be associated with pemphigus (Ambiel and Roselino et al., 2014).

## 6.4. Concluding Remarks

Despite the difficulty of associating BP with other disease, it is inevitable that one is more contested than others. For instance, a considerable number of BP patients have been associated with neurologic disorders; however, it has also been much discredited. It is still not clear if the association is merely casual.

This chapter, onde agauin, reinforces the fact that it is necessary further research about the underlying mechanisms between these associations. Also, Very little is known about BP association with psychiatric diseases, and many cases have been reported.

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# Chapter VII

**Triggers – What Could Possibly Cause This?**

## 7.1. Bullous Pemphigoid Inducing Factors

A great majority of cases don't have an obvious precipitating factor that could induce BP. However, in some cases, with a meticulous clinical history, it is possible to detect some agents that could be responsible for inducing this disease.
It was once observed that a recognizable factor that could precipitate BP was present in no more than 15% of patients, which once again lead us to think that inducing factors can only account for a minority of all the BP cases (Venning and Wojnarowska, 1995). Moreover, since the mechanisms that could be responsible for BP are unknown, it is possible to assume that most are related to factors that could locally disrupt the basal membrane zone of the skin (Lo Schiavo, et al., 2013).

### 7.1.1. Drug Induced

The term drug-induced bullhous pemphigoid (DIBP) used to describe cases of BP with clinical, histological and immunopathological characteristics that are similar to idiopathic diseases' characteristics. These are induced by the systemic ingestion, or even by local use, of some drugs. Furthermore, a genetic susceptibility might play a role, because only few people develop the disease after the intake of certain offending drugs (Kanahara and Agrawal, 2016; Vassileva, 1998).
The course of this BP is not uniform, and it's possible to discern two types: an acute and a chronic. The acute self-limited form shows a definitive resolution after withdrawal of responsible drug, and with or without steroid therapy. The chronic form usually seems to be precipitaded by the drug administration and, during time course, ends up assuming the classic BP characteristics (Ruocco and Sacerdoti, 1991). Since it is possible that drugs can act as triggers in BP patients having genetic susceptibility, two main mechanisms are implicated: the modification of the immune response and the alteration of the antigenic properties of the epidermal basement membrane. This means that the production of autoantibodies against the basal membrane zone can be induced by drugs, which act like haptens that bind to proteins in lamina lucida and change their antigenic properties. Moreover, drug can also stimulate an autoimmune response by structurally altering molecules and uncovering hidden epitopes (Ruocco and Secerdoti, 1991).
Systemic and topical treatments can equally induce BP, and they can be grouped according to their chemical structure. The majority of systemic drugs has or releases sulfhydryl groups (thiols: penicillamine (Popadic et al., 2009), captopril (Mallet et al., 1989), penicillin and derivates (Hodak et al., 1990; Wozniak et al., 2006). The sulfhydryl groups' key role could be within the drug precursor or within a catabolized metabolite. It seems that the biochemistry of free sulfhydryl groups is crucial for drug-induced BP (Walsh et al., 2005). Thiol group seem to allow the molecule to combine with proteins in the lamina lucida, acting as a hapten, and resulting in the autoantibody formation to BMZ proteins. Another explanation is that certain sulfur-containing drugs could cause a dermo-epidermal split without immune mediation. Besides, there are some sulfur-containing drugs (like penicillamine) that could interfere with Treg cells, which will cause a decrease in the suppressor cell activity, with subsequent exacerbated production of different autoantibodies against the BP antigens (Durdu et al., 2011; Popadic et al., 2009).

More drugs can be associated, such as drugs that contains a phenol ring, some cephalosporins and aspirin (Durdu et al., 2011) and non-thiol non-phenol drugs – angiotensin-converting enzyme inhibitors other than captopril (Smith et al., 1993), majority nonsteroidal anti-inflammatory drugs (Laing et al., 1998), nifedipine (Ameen et al., 2000; Brenner et al., 1999).

The suspicious that a common drug like aspirin could trigger BP led to believe that, maybe, this drug act as a hapten, which alters the antigenicity of the laminda lucida or even that could maybe attach to a cell site and lead to autoantibodies' formation (Durdu et al., 2011).

Recently, several BP cases were reported to be associated with dipeptidyl peptidase-IV (DPP-4) inhibitors, which are used for type 2 diabetes mellitus (Aouidad et al., 2013; Attaway et al., 2014), more specifically, cases associated with linagliptin and vildagliptin-metformin use (Mendonça et al., 2016; Pasmatzi et al., 2011; Sakai et al., 2016). Taking into account the cases of vildaglipin-induced BP, awareness during gliptin therapy could prevent cases of BP development (Keseroglu et al., 2016). But until now, the clinical and immunological characteristics of DPP-4 inhibitor related to BP have not been clearly elucidated.

Interferon-gamma (IFN-γ) that is released from lymphocytes test might help physicians to better diagnose drug-induced pemphigus (Brenner and Goldberg, 2011). This kind of diagnosis is based on skin reactions resolution of the eruption, upon cessation of the drug suspected by the assay (Goldberg et al., 2004; Halevy et al., 2005). The IFN-γ released from the patient's lymphocytes is evaluated after *in vitro* incubation with and without the suspected drug. Thye patient's lymphocytes are separated from heparinized venous blood. Ultimately, the IFN-γ can be detected by ELISA assay. The IFN-γ is used to detect an immune sensitization to a culprit drug and identify it among all different drugs that the patient is taking (Brenner and Goldberg, 2011).

### 7.1.2. TNF-α Blockers

Treatment with TNF-α blocker has been reported to be associated with many cases of BP (Bordignon et al., 2009; Kluk et al., 2011; Toosi and Bystryn, 2010[b]). Tumor necrosis factor-alpha (TNF-α) is an important regulator of inflammation and it's responsible for the increased production of pro-inflammatory molecules, such as IL-1, IL-6, IL-8, etc, and adhesion molecules (e.g. intercellular adhesion molecule-1, P-selectin, E-selectin) (Wajant et al., 2003). Likewise, it is well known that TNF-α can promote apoptosis. Therapy with TNF-α antagonists is proven to be effective in treating inflammatory disorders (Esposito and Cuzzocrea, 2009; Victor and Gottlieb, 2002). TNF-α down-regulation by TNF- α blockers (e.g., adalimumab and etanercept) could be implicated in BP development on patients with a propensity for this disease. It is necessary to outwit BP in patients under treatment with biological agents, such as efalizumab therapy (Duong et al., 2010; Wilmer et al., 2016).

TNF antagonists are used in patients with psoriasis, with shown efficiency, so it can be pertinent to use this therapy in BP patients, especially when BP coexists with psoriasis (Cusano et al., 2010; Yamauchi et al., 2006).

What is most important to retain about this topic is that, since this therapy reveals efficacy in other immunobullous disorders, the efficacy of this same therapy in BP should be established too.

### 7.1.3. Contact Pemphigoid

The external use of some preparations of the skin or mucous membrane has been reported to induce cases of BP. These preparations have such an irritating effect (for example benzyl benzoate) or allergic contact hypersensibility (like 5-fluorouracil) that have been proposed as a triggering effect for BP (Bart and Bean, 1970; Piletta et al., 1994; Stransky et al., 1996; Vassileva, 1998).

### 7.1.4. Vaccinations Contribution

Not many cases of vaccinations have been implicated in inducing BP, although there's still some cases correlated with it: anti-influenza vaccine and swine flu vaccination (Mérida et al., 2005; García-Doval et al., 2006; Walmsley and Hampton, 2011), tetanus toxoid booster and tetracoq vaccine (Baykal et al., 2001). Being the vaccination so widely used and carefullu developed, it is unlikely that they could activate anti-BMZ antibody production. But, as Lo Schiavo et al. questioned: why have reports of BP triggered by vaccination been rarely reported when vaccination practice is so common? Maybe, vaccination only induces BP in patient's genetically prone to it, with an immunologic predisposition or even with subclinical BP (Lo Schiavo et al., 2013).

### 7.1.5. Induction by Physical Agents

Over time, many studies have been connecting BP induction with ultraviolet (UV) light – UVB or psoralen with UVA – radiation therapy (RT), thermal or electrical burns, or surgical procedures (Dănescu et al., 2016; Khandpur and Verma, 2011; Korfitis et al., 2009). Moreover, in these cases, BP could become generalized or localized to the injuried site – immunocompromised district (Ruocco et al., 2009).

**Radiation Therapy Involvement** It is within the female population with breast cancer, where the majority of BP radiation therapy induced cases have been registered (Kluger et al., 2016). In a cohort with twenty nine breast cancer patients, about 72% developed BP (Nguyen et al., 2014). This could mean that maybe radiation has the ability to change antigenic properties and induce autoantibody production by altering the BMZ, unmaking antigens. Or maybe it is possible that patients have circulating low titre anti-BMZ antibodies, and all this together with tissue damage caused by RT can enhance deposition of these autoantibodies, through alteration of blood vessels' permeability. It is known that radiation alters MMP-9 values and growth factors (such as VEGF) and that it can cause an induction of local modifications of the immune system. It is possible that, sooner or later after RT, BP appears and after the first onset in the irradiated area, it could become generalized. This fact could be explained by the epitope spreading. Then, an immune response spreads to cover different sites on the same autoantigen and even different autoantigens (Binitha et al., 2014; Campa et al., 2016; Isohashi et al., 2011; Mul et al., 2007; Srifi et al., 2011).

**Ultraviolet radiation** Experiments with organ-cultured normal human skin revealed that BP antigen could probably be susceptible to UVB irradiation, and this could lead to configurational changes in these antigens (Muramatsu et al., 1994). Also, this kind of event could occur with UVA therapy too (Washio et al., 2005), in which serum levels of IL-1 raise leading to polyclonal activation of B cells. It has been demonstrated that many BP cases often appeared after anti-psoriatic treatments – sun exposure, PUVA, UVA and UVB therapy (Barnadas et al., 2006; Caca-Biljanovska et al., 2016; Kao et al., 2008). Washio et al. reported a case of a thirty five year old white man diagnosed with psoriasis that developed blisters on his extremities, after UVB phototherapy all over the body. This could be an indicator that maybe UV radiation alters BMZ antigenicity, exposing or releasing altered antigens. Furthermore, these antigens could stimulate antibody formation against BMZ (Washio et al., 2005). With all these findings it's possible to infer that UV therapy could be the most likely trigger of BP.

**Photodynamic therapy** Photodynamic therapy (PDT), also called photochemotherapy, is a form of phototherapy involving light and a photosensitizing chemical substance, used clinically to treat psoriasis, atherosclerosis and malignant cancers, in particular skin (Chen et al., 2002; Morton et al., 2013; Saini et al., 2016). An eighty eight year old man was diagnosed with BP, after topical PDT for a large Bowen's disease on his left cheek. There is only one more case reported of localized BP associated with PDT (Morton et al., 2013; Rakvit et al., 2011). So, it is possible that PDT can induce BP in predisposed individuals or, for instance, cause damage in the basement membrane and antibody formation.

**Burns** Thermal or electrical burns are known to be one of the physical BP inducers. Usually, BP develops in the affected areas and, later on, extends to other skin areas. However, it is important to discern BP lesions from physical injuries. One factor that could help to link a burn to BP occurrence is that, for example, patients don't have a relapse once the triggering factor (electrical ou thermal burn) is eliminated (Bachmeyer et al., 2010; Balato et al., 1994; Damevska et al., 2014; Kluger et al., 2011; Morita et al., 2015; Xu et al., 2008).

**Surgical Procedures** In this case, BP is confined to the accidentally traumatized area (Ruocco et al., 2009). The injuries that have an association with BP, often appear after different types of surgical procedures, such as percutaneous endoscopic gastrostomy (PEG) (Nozu and Mita, 2010), urostomy (Batalla et al., 2011; Torchia et al., 2006), surgeries near the venous site in hemodialysis patient (Yesudian et al., 2002), among others (Neville and Yosipovitch, 2005).
These sorts of events may happen because of the damage to the dermal-epidermal junction and antigen exposure, which triggers an immune response. The skin around the PEG tube is frequently inflamed by gastric juice and chronically irritating skin can induce activation of the immune system, and this could mean a huge difference between PEG and tracheal tube, since traqueal tube has no association with BP (Nozu and Mita, 2010).

### 7.1.6. In Transplant Patients

This association is rare to happen, however, there are a few cases reported in the literature. The most important association is made between BP and renal transplant. Usually, the patients responded only to systemic corticosteroids and the skin lesions in some cases improved after graft removal. This might be a strong suggestion that renal allograft has something to do with BP pathogenesis, maybe because of the autoantibody production due to renal allografts. This production could be the result of chronic allogeneic stimuli. Also, it is possible that an immune cross-reactivity between skin and the kidney has an important role. Because sometimes antigenemia due to chronic rejection can occur in transplantation, this might have contribution to BP pathogenesis. It is noteworthy that, since renal transplantation patients take immunosuppressants to avoid rejection, these drugs may also have something to do with BP development. Tregs play an important role in autoimmunity and graft tolerance, since Th and B cells are directly suppressed. Tregs are found in decreased levels in peripheral blood, after tracolimus traeatment (Abou-Jaoude et al, 2005; Chen et al., 2009). Tracolimus, also called FK-506, is an immunosuppressive drug used mainly after allogeneic organ transplant to lower the risk of organ rejection (Kino et al., 1987). Since, Tregs need low-dose IL-2 (responsible for promoting development and proliferation of T cells) signals in order to survive, tracolimus acts inhibiting the production this interleukin, so suppression of Tregs numbers occur. The Tregs deficiency, indirectly contributes to autoantibody induction (Chen et al., 2009). Also, a case report described a case of BP after liver transplantation, for liver failure, in a child with Coomb's positive autoimmune hemolytic anemia and giant cell hepatitis (Kerkar et al., 2006). A case of a twenty eight year old woman with tuberous sclerosis complex (TSC) diagnosed with BP was reported. And despite the lack of association between BP and TSC in this case, it is important to say that this patient underwent renal transplantation after bilateral bephrectomy, so, once again, we could be in the presence of another case diagnosed with BP after renal transplantation (Mandel et al., 2016).

### 7.1.7. Infections Association

Over time, investigators seem to find a link between BP pathogenesis and some infections, in particular herpes virus infections. Patients with BP have been reported having human herpesviruses (HHV), such as cytomegalovirus, Epstein-Barr and HHV-6. Detection of these viruses seems to happen in vesicular fluid, while in the patients'serum the detection of antibodies to HHV-8 occurs. However, one of the viruses' characterisitcs makes us question its etiopathogenic role – their latency in specific set of cells (Drago et al., 2005). Other viruses have also been implicated in BP induction, such as hepatitis B and C (Sagi et al., 2011). Also, Torque Tone virus has already been reported with high incidence in BP patients' sera, however, the role in the disease is still not very clear (Blazsek et al., 2008). A virus can induce autoimmune disease, by damaging the infected cells. Furthermore, the new antigens can derive from the incorporation of fragments of the cell membrane into the envelope of the virus. Another possibility is that viruses may share epitopes with its host, which could lead to the development of cross-reactive autoantibodies – molecular mimicry. Additionally, viruses could also activate polyclonal B cells to produce autoantibodies, and interfere with T cells and their lymphokine production (Drago et al., 2005).

## 7.2. Pemphigus Vulgaris Inducing Factors

Similar to what happens with BP, in PV there is no underlying agent that could induce this disease, leading us to think that this could happen spontaneously. But, in fact, if we meticulously study the patients' medical files we can find some possible associations.

### a. Drugs: feasible inducer

The drug intake represents the most common cause of triggered pemphigus. The likely drugs to cause disease are thiols and phenols, among other non-thiol and non-phenol drugs (Ruocco et al., 2013[a]). Drugs containing thiol group are indicated as causative in precipitating a real antibody-mediated PV, since the infiltration of thiols in keratinocytes leads to changes in the molecular structure and alters the antigenic conformation of membrane desmogleins. Also, thiols could cause an immune imbalance and amplifying the acantholytic self-injury (Ruocco et al., 1993; Ruocco and Ruocco, 2003). The most common thiol drugs triggering pemphigus are penicillamine, captopril, and penicillin and derivates (Ruocco et al., 2013[a]). Concerning the non-thiol and non-phenol drugs, enalapril (angiotensin-converting enzyme (ACE) inhibitors), nonsteroidal anti-inflammatory drugs, nifedipine and, most important, biologic modifiers of the immune response, like vaccines, interferons (IFN) and other cytokines are the most likely PV trigger (Ruocco et al., 2013[a]). There are several reports of PV patients associated with influenza, tetanus and diphtheria vaccinations, some with a fatal outcome (Ruocco and Ruocco, 2003). There is one study that reported pemphigus antibodies in the sera of non-pemphigus patients that did IFN-α therapy for awhile (Baroni et al., 2011).

A case report refers a sixty three year old white man that suffered from a relapse of PV after topical application of ingenol mebutate gel, representing the first case of this kind. Russo et al. postulate that, maybe, this happens because of the proinflammatory activity of this topical drug (Russo et al., 2016).

Antihypertensives are also suspected to trigger/sustain PV/PF. About 62.50% of AH positive PV patients took ramipril and about 93.33% of AH positive PF patients took enalapril, both potentially noxious drugs for AH (Pietkiewicz et al., 2015). However, it was reported that the calcitriol, a hormonally active vitamin D metabolite, can protect keratinocytes from captopril-induced cell detachment and apoptosis (Zeeli et al., 2011).

### b. Virus Part

As mentioned earlier for BP, herpes virus infections are indicated as possible inducers in PV (Ruocco and Ruocco, 2003). A study with 20 PV patients reported Herpes simplex (HSV), Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV-6) circulating in lymphomonocytes and in some patients' skin lesions. This could indicate that herpetic infections might induce upregulation of humoral and cellular pro-inflammatory factors, as aforementioned (Tufano et al., 1999). However, viral infections only induce PV ocassionally or, perhaps, only exacerbate the autoimmune disorder in genetic susceptible patients.

### c. Physical Triggers

When we talk about physical agents that could possibly induce pemphigus, there is no way to dissociate from the physical agents implicated in BP cases, since there is an obvious resemblance. Agents like sunburns, ionizing radiation, thermal and electrical burns, as well as surgical and cosmetic procedures, are porved to be inducers in individuals PV' predisposed (Ruocco et al., 2013[a]).

Again, similarly to what happens in BP, the UV radiation is the main cause in pemphigus induction. Therapy with ionizing radiation can also trigger PV, as previously reported in literature (Robbins et al., 2007). The thermal and electrical burns that induce pemphigus lesions, similar to what happens with ionizing radiation, usually lie within or in the adjacent tissue of burned areas. Later, they tend to extend to other regions of the skin and, sometimes, to oral cavity (Tan et al., 2006). It seems that there is no relation between PV and any specific surgical procedure, since it manifestated following a series of various procedures (electrosurgery, hair transplantation, facelift, etc). Nonetheless, dermobrasion and chemical peels seem to favour acantholysis in PV susceptible individuals, maybe because the procedures include chemicals containing phenol (Ruocco et al., 2013[a]).

### d. Allergens Playing a Part

Allergic contact dermatitis (ACD) can be a mediator in PV exacerbation, through some chemical sensitizers, such as photographic developing, dry cleaning, industrial solvent work, gardening, and more. There are some reported cases of PV after contact with pesticides, for instance (Vozza et al., 1996). Brenner et al. conducted an international survey that reported the pesticides' exposure as a potential cause for an increased risk for PV (Brenner et al., 2001). Another interesting fact is that PV is more common in nonsmokers than in smokers. It seems that nicotine, as a cholinergic agonist, acts like a protection factor against cell-to-cell detachment (Valikhani et al., 2007[b]).

### e. Emotional stress

Cytokines and neurotransmitters are responsible for the communication between nerve fibers and cutaneous cells, which represent a unique neuroimmunocutaneous system (Misery, 1997), and this well-designed system is responsible for a pathogenic link between emotional stress and an autoimmune skin disorder, with some cases being reported. Some of these cases correlated the onset of the disease in patients with personality disorders experiencing a stressful event (Cremniter et al., 1998; Morell-Dubois et al., 2008). The most prevalent stress events causing induction of PV are environmental disasters, war, terrorism, relative's death and sexual aggression (Ruocco et al., 2013[a]).

## 7.3. Pemphigus Foliaceus Inducing Factors

As mentioned earlier, the affinity in the development of IgE and IG4 antibodies could transpire due to the sequential isotype switch from IgM over IgG4 to IgE. The switch of both isotypes is promoted by Th2 cytokines – IL-4 and IL-13. The chronic antigenic stimulation induces secretion of IL-10 that shifts the balance of the class switch towards IgG4 (Jeannin et al., 1998). The imbalance of IgE and IgG4 brings benefits to patients with allergies, but the same cannot be said about autoimmune skin diseases such as FS,

in which this process could trigger a massive IgG response that could lead to generation of pathogenic IgG4 autoantibodies. Moreover, an environmental allergen could probably trigger IgE responses in FS, and the antibodies directed against this allergen cross-react with epidermal Dsg1 (Qian et al., 2016).

A recent study searched for triggering environmental antigens, in patients with EPF, *Fogo Selvagem*. It was suggested that the exposure to hematophagous insect bites could possibly constitute a precipitating factor for FS development. In endemic regions, FS patients and healthy individuals demonstrated IgM autoreactivity response from early childhood, that later restricts to IgG4, in FS patients. So, this search for triggering environmental antigens, led to a discovery in which IgG4 and IgE autoantibodies, from the patients with FS, cross-react with a salivary antigen from sand flies, which proves that these antibodies evolve from the naïve B cells. This also demonstrates that this non-infectious environmental antigen could be an initial target of the autoantibodies response in *Fogo Selvagem* (Qian et al., 2012, 2016). Qian et al proposed that there is a possible mechanism that might trigger the development of anti-Dsg1, as seen in **Figure 19**, by environmental antigens. This mechanism could be LJM11, a sand flies salivary gland antigen (SGLL) component recognized by humans. Probably, this component stimulates naïve B cells to undergo class switch in order to generate an initial IgE response directly agains the LJM11 component. Since there is cross-reactivity between the epitopes of LJM11 and Dsg1, the initial antibody response – IgE response – also rests to Dsg1. Moreover, the chronic stimulation of LJM11 antigen and consequent production of IL-10 may induce the development of IgG4 antibodies, that also reacts to both LJM11 and Dsg1. There are two outcomes expected fot those IgG4 antibodies: are pathogenic in FS or trigger further developmente of IgG4 autoantibodies via epitope spreading (Li et al., 2003; Qian et al., 2016).

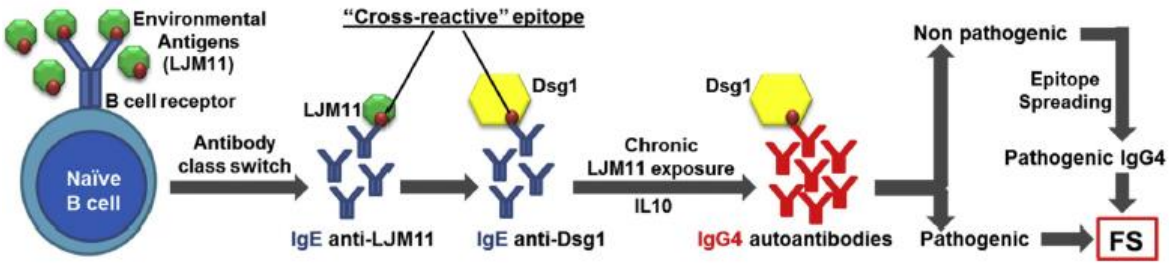


**Figure 19 – Environmental antigens, like LJM11, triggering the development of cross-reactive antibody response and subsequent FS, in individuals prone to (from Qian et al., 2016).**

It is common knowledge that the use of topical herbal supplements to improve health has had great success in the last decades. One of these supplements is the popular St. John's wort, or *Hypericum perforatum*. However, these kind of "homemade medicine" can trigger multiple adverse effects, with a well-known photosensitizing and, possible, immunomodulatory effect (Bedi et al., 2002; Klemow et al., 2011). This flowering plant has a rate of 50% probability to cause adverse effects, which are mild and transient (Ernst, 2000). Even though we know that this herbal drug has its own adverse effects, such as allergic reactions, gastrointestinal disturbances, among others, the mechanism of action is still unknown, and so this drug can potentially cause autoimmune diseases, since it may

increase activity of macrophages, neutrophils, natural killer cells, and proinflammatory cytokines, like IL-1 and TNF-α (Ernst et al., 1998; Lee and Werth, 2004). Photoxicity is a very common side effect of this herbal drug, which can induce pruritic skin rash (Imbernón-Moya et al., 2016). A case of a thirty seven year old woman with PF was temporarily associated with the herbal drug, after she started taking St. John's wort, one week before the appearance of skin lesions. However, the cause-effect cannot be fully established, so further studies are needed (Imbernón-Moya et al., 2016).

### 7.4. Nutrition May Interfere - Transversely Speaking

Factors derived from our daily diet have been implicated to play a part in pemphigus induction, with a variety of substances that are present in different foods that we regularly eat being the culprit. These substances are believed to play a role in causing the induction of some pemphigus cases, more properly in patients with genetic predisposition (Lakdawala et al., 2013).

Some molecules with active thiol groups could contribute to induce pemphigus. For example, some plants, like those belonging to the *Allium* group, contain active compounds with stable disulphide and thiol groups. The *Allium* group includes some important vegetables such as onions and garlic, so it is suggested that this kind of food can contribute for this induction (Brenner and Wolf, 1994; Chorzelski et al., 1996; Ruocco et al., 1996).

Also, a gluten free diet has been indicated to possible improve condition of pemphigus and BP patients, in cases where there is underlying gluten sensitivity (Fedeles et al., 2010). Antigliadin antibodies (AGA) have already been described in pemphigus patients, in the past (Kumar et al., 1992).

It seems that diet doesn't have a direct involvement in BP induction, since no triggers are suspected (Lakdawala et al., 2013). A case of dyshidrosiform pemphigoid induced by nickel in diet was reported, which was resolved by a nickel-free diet. However, it is noteworthy that some BP patients have gluten intolerance, proven by the presence of antigliadin antibodies in patients' sera. This, once again, seems to be resolved with a gluten-free diet (Fedeles et al., 2010).

**We cannot say the same for pemphigus.**

A forty nine years old man gives us the perfect example of the consequences from heavy garlic consumption, which worsened pemphigus already diagnosed. In this case, the garlic-free diet eased symptoms of the disease. Also, the recurrence occurred due to ingestion of a garlic meal (Ruocco et al., 1996). Another case was reported with oral lesions associated with leek ingestion (Chorzelski et al., 1996).

A study made *in vitro* reported that three garlic' compounds – allylmercaptan, allylmethylsulfide and allylsulfide – caused acantholysis in skin specimens at 6mM concentration (Brenner et al., 1995).

Another compound that has been implicated in pemphigus induction is phenols. There are case reports of patients diagnosed with pemphigus due to topical applications of phenol-containing chemicals (Goldberg et al., 2001; Kaplan et al., 1993). India has the higher rates of younger age cases diagnosed, which can be explained by the high consumption of foods with high levels of phenols, like mango, cashew nuts and black peppers (Brenner

et al., 2000, 2006). Tannins-containing foods have also been suggested to play a role in pemphigus induction. This plant's polyphenolic compound is present in some daily foods and drinks, such as mango, guarana, raspberry, cranberry, blackberry, avocado, peach, ginger, ginseng, tea and red wine (Tur and Brenner, 1997, 1998). This compound may be behind the high occurrence of endemic pemphigus in Amazonian Brazil (*Fogo Selvagem*), due to increased amounts of this compound being dissolved in the water systems that serves the Amazonian natives (Tur and Brenner, 1997). And since food with high tannin concentrations is also widely consumed in India, this may cause a high incidence of pemphigus there, similarly to what happens with phenols. There is a report of an *in vitro* cultured skin to which was added tannic acid that produced many cytotoxic effects, one of them being acantholysis (Brenner et al., 2000). By high-performance liquid chromatography (HPLC) the levels of tannic acid (TA) and gallic acid (GA) in blister fluid of 4 groups of patients were measured. The groups were made according to dietary habits, such as regular diet, diet rich in tannins, diet free of tannins and pemphigus patients. The group with a diet rich in tannins demonstrated high tannin metabolites in the skin. In this study, an *in vitro* acantholysis system revealed that TA is able to cause acantholysis (Feliciani et al., 2007).

Isothiocyanates, chemicals derived from hydrolysis of glucosinolates, are found in vegetables of the *Cruciferae* group, which includes mustard, broccoli, turnip, cabbage and cauliflower. This chemical may contain groups of allyl, benzyl or phenyl and they could be immunologically active and behave like thiol-containing drugs (Wittstock and Burow, 2007). The allyl isothiocyanate is the main compound of mustard oil, known to cause blistering of mucous membranes (Le, 1964). This kind of components could be implicated in the induction of pemphigus; however, to date no cases of pemphigus due these chemicals have been reported.

Phycocyanin is a blue pigment protein present in algae like *Spirulina platensis*. These "algae" are a cyanobacteria sold as a dietary supplement (Lupatini et al., 2016). Also, it was reported that spirulina has as an immunomodulatory effect that mainly stimulate the innate immune system (Hirahashi et al., 2002). There are case reports with this alga and immunoblistering disorders. One is from a fifty seven year old man with chronic PV that experienced a severe extension after intake of spirulina supplements, while another case is from an eighty two year old healthy woman who experienced a mixed immunobullous disorder with features of PV and PF, 1 year after she started taking spirulina supplements. In the first case, the extension improved after treatment and discontinuation of spirulina intake, and in the second case the woman recovered (Lee and Werth, 2004; Kraigher et al., 2008). Very recently, Sarre et al. found an association between BP and hypovitamosis in older patients, in cases with increased comorbidity cluster. We are now aware of the role for vitamin D in the regulation of immune responses (Baeke et al., 2010). Only a few studies had already focused mainly in the association between low serum 25-hydroxyvitamin D (25OHD) concentrations and BP (Marzano et al., 2012, 2015; Tukaj et al., 2012), all with somewhat contradictory conclusions. Marzano et al., both in 2012 as in 2015, reported a significant association and Turkaj et al. reported no association at all. The big difference in Sarre et al. study is the comorbidity burden, which seems to influence this association, so this study is consistent with previous evidence of the association between vitamin D and autoimmune disorders (Sarre et al., 2016).

## 7.5. Concluding Remarks

There are already some tools that help physicians find out what possible causes of induction of these three ABD.

Along with all the possible triggers that have already been described, it seems that pregnancy can exacerbate pemphigus but, often, to a mild degree. On the other hand, pemphigus seems to cause a higher incidence of abortion (Lin et al., 2015).

Also, there are many suggestions that can help the patients to avoid the blistering or to find an adequate treatment. For instance, in patients that are genetically prone to pemphigus, it would help if they could distance themselves from the environmental triggering factors, helping in the management of the disease. This could also improve the efficacy of treatments, avoid relapses and, most likely, result in a cure.

As mentioned, FS is the pemphigus form with the strongest link with the environment and this fact could result in the possibility to use FS as a great model to study the impact of environmental antigens in the autoimmune disease development.

Another concern is the wide use of herbal supplements that could possible trigger these ABD. Maybe more scientific studies about this matter are needed, in order to prevent future blistering diseases cases. These immunostimulatory supplements exacerbate pre-existing autoimmune disease, like pemphigus, or maybe precipitate the disease in individuals with certain genetic predisposition. Either way, it might be necessary to study its influence. It was also alarming to see that there are some cases of vaccinations that could induce disruption of the membrane architecture, leading to the generation of anti-BMZ antibodies. And since this relationship remains obscure, it could be interesting to study and deepen this connection.

Also, and since the link between BP and certain drugs is not clear, it might be useful to screen BP patients for drug induction, so it could be possible to prompt discontinuation of the culprit drug. This cessation could result in a more quick and effective recovery of the patient. Studies have been made in order to find an evident association between BP and drug intake, like Tan et al. (Tan et al., 2016).

Moreover, for all that was mentioned above, PDT should be added to possible BP triggers.

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# Chapter VIII

## Genetic Susceptibility

By now, it is well established that pemphigus has a strong genetic predisposition; however, the inheritance is still not clear and seems to be rare. This fact could happen because more than one gene causes the disease or maybe because of additional endo and exogenous contributing factors (Gazit and Loewenthal, 2005). The real proof of this genetic event is the high incidence in Jews, mainly among Ashkenazi (Krikler, 1970). One of the first reports about this genetic event demonstrated a major association between pemphigus and HLA in Jews, more specifically HLA-A10 (Krain et al., 1973). A study from 1979 already reported a prevalence of HLA-DRW4 in 91% of Jews with PV, reporting an extremely high incidence and association, in what the authors classified as an "HLA concordant" disease in Jewish patients (Park et al., 1979). The reason why Jewish population is so associated with pemphigus is their isolation, intermarriage, proselytism and genetic drift (Loewenthal et al., 2004).
Over the years there has been a great interest in genetic associations with pemphigus pathogenesis. The crucial link lies within the major histocompatibility complex (MHC) genes, essentially the HLA class II (Brochado et al., 2016).
There are many populational studies that correlate HLA class II alleles with PF and PV.

### 8.1. Jewish community

There is a tremendous interest in genetic studies relating the Jewish community and pemphigus disease. The first genetic association in Jewish patients comprised HLA-A26, HLA-B38, HLA-DRB1*0402, HLA-DQB1*0302. These haplotypes are the most commonly found in Jewish groups (Mobini et al., 1997).
The haplotypes HLA-DRB1*0402, DQB1*0302 have been commonly associated with Jewish PV patients worldwide, whereas the haplotypes HLA-DRB1*1401/ HLA-DRB1*1404, DQB1*0503 have been linked to non-Jewish PV patients, generally speaking (Fridkis-Hareli, 2006).
It is important to keep in mind the strong association between PV and PF to HLA class II genes, as was already mentioned. However, a study found out that, HLA-G in Jewish communities is also strongly associated with the disease (Gazit et al., 2004). This is interesting since this gene, besides belonging to nonclassical HLA class I, is suggested to have a crucial role in immune tolerance in pregnancy, being expressed in the placenta (Menier et al., 2000). It is still not clear exactly how HLA-G can affect PV pathophysiology. However, it is known to participate in immunological cascades that lead to production of anti-Dsg antibodies (Gazit et al., 2004).
Hopping to find some genetic association besides the HLA class II genes, a study used sixteen microsatellite probes to scan the entire MHC region and screen samples from thirty wight PV patients and seventy six healthy controls. Maerkers were, again, reported to be associated with the class II region but the HLA-A region was also mapped, highly related to PV. So these results give an insight to the importance of gene, or genes, other than class II, in initiation of the autoimmune cascade, meaning that the activation or the suppression of these genes could be a trigger mechanism to start this cascade (Slomov et al., 2003). Again, in 2005, another study tried to find association between PV and HLA class I TAP genes, using thirty seven patients Jewish Israeli and thirty seven healthy Jewish Israeli. The two risk alleles TAP2*C and TAP2*D were identified and estimated to be 37.8% in patients and 5.3% in controls. So, it was possible to conclude that TAP2

genes are responsible for susceptibility in developing PV (Slomov et al., 2005). Also, an analysis to one hundred and fifty five pemphigus patients, from a medical centre in Israel, revealed that only 37% of the sample represents Non-Ashkenazi Jews, another niche of great interest to study. This study reinforces the link between pemphigus and HLA genes (Mimouni et al., 2008). The Ashkenazi Jews have been studied because they represent the highest prevalence of pemphigus vulgaris, for a long time now (Brautbar et al., 1980). For instance, Seidenbaum et al., reported that of the patients studied in the cohort, about 75% were Ashkenazim (Seidenbaum et al., 1988).

## 8.2. Population Studies

### 8.1.1. Africa

**Tunisia** Genetic studies were also conducted for Tunisian patients. A study with 90 PF patients found that DRB1*0301 allele confers susceptibility to Tunisian PF, which is surprising since DRB1*03 wasn't associated with the endemic or sporadic form of PF. The cohort was divided in two groups, one with patients from the north (sporadic PF form) and on with patients from the south (endemic PF occurence). They concluded that DRB1*03 allele is the main susceptibility allele of the Tunisian EPF. Moreover, the DRB1*04 allele is the most important conferring susceptibility with higher significance in the south group than in the north group. The differences that this study reported in north and south group, suggest that PF might occur in two forms, in Tunisia, a sporadic and an endemic form (Abida et al., 2009).

**Morocco** Attempting to find a link between high prevalence of pemphigus and a specific genetic factor in morocco, a study with a cohort of fifty two pemphigus Moroccan patients (seventeen PV and eleven PF) was conducted. An increase in DRB1*04, DRB1*14 and DQB1*03 allele frequencies and a decrease of DRB1*15 and DQB1*06 allele frequencies was found. However, these values are similar to those commonly found among other populations. Furthermore, the HLA-DRB1*15/DQB1*06 haplotype revealed protective effects in this population whereas DRB1*04/DQB1*03 and DRB1*14/DQB1*05 haplotype induced susceptibility to pemphigus disease. These results reinforce a genetic predisposition to pemphigus. Nonetheless, in this population the genetic factors continue to not explain the high prevalence of disease in Morocco (Brick et al., 2007).

### 8.1.2. Europe

**Spanish** González-Escribano et al. reported a study with 26 Spanish Caucasian with PV that was HLA typed. Twenty three of them were HLA-DR4 and twenty one had the DRB1*0402 allele, so the frequency of this allele was 81%. They also noticed another fact: HLA-DR13, which is present in 27% of Spanish general population, wasn't present in these PV patients. It was possible to conclude that in Spanish population, PV is mostly associated with HLA-DRB1*0402 and, on the other hand, DRB1*13 seems to be conferring protection to Spanish population (González-Escribano et al., 1998). Another study investigate Jews lived in Spain for more than 1500 of years. Many of these Jews converted to Christianity, which favoured admixture with the Spanish. This study proves

that the same HLA haplotype – DRB1*0402 and DQ0302 – is present in both Jewish and Spanish PV patients. This suggests that they had the same founder (Loewenthal et al., 2004).

**Italy** A study with thirty two Italian, sixteen of which Sardinian, PV patients reported two PV susceptibility haplotypes: HLA-DRB1*0402, DQA1*0301, DQB1*0302 and HLA-DRB 1*1401, DQA1*0104, DQB1*0503, being thah the first haplotype was the most prevalent among Sardinian patients. They concluded that the strength of the allele associations to PV lies on DRB1*0402 and DQB1*0503 alleles. Similar to what happens to Spanish population, there is an allele HLA-DR3 that seems to confer protection against PV (Carcassi et al., 1996).
Another study involving eighty seven Italian patients, of which sixty one had PV and twenty six had PF, was conducted. This study revealed an increase of DRB1*04 and DRB1*14, in both PV and PF patients. Both pemphigus forms demonstrated a high association with DRB1*1401. They also concluded that, in these cohort, PV and PF shared DRB*1401 and DQB1*0503 susceptible HLA alleles. Also, they determined that DRB1*0402 is only associated with PV (Lombardi et al., 1999).

**France** French population was also studied, represented by a cohort with fifty seven patients, thirty seven of them with PV and twenty with PF. The results concerning PV were in line with previous studies from other countries. However, concerning PF, it was demonstrated that DRB1*01012 and DRB1*0404 are susceptible molecules for the French population (Loiseau et al., 2000).

### 8.1.3. Asia

**Turkey** When the Turkish population was studied for the HLA subtypes, it was possible to conclude, in a cohort of twenty five PV patients and one hundred nd thirteen healthy controls, that 68% of the patients had the allele HLA DRB1*04, against 30.97% of the controls, making it more prevalent in infected patients. The allele HLA DRB1*14 was also expressed with an increased frequency in the patients, with 32% against 8.85% in the control group. Also, the frequency of the haplotypes HLA DRB1*04/HLA DRB1*03 and HLA DRB1*14/DQB1*05 was significantly higherin the PV patients of this cohort. It was not possible to identify alleles or haplotypes that could confer some form of protection to PV in this cohort. So, all of these alleles and haplotypes described are indicated to be responsible for genetic susceptibility in Turkish population (Tunca et al., 2010).
**Iran** A group of fifty two PV Iranian patients revealed that HLA-DRB1*04 and HLA-DRB1*1401 alleles are two major PV susceptibility factors, in Iranian population. This cohort only covered non-Jewish patients (Shams et al., 2009).

**Pakistan** Pakistani population was also studied and it was found a strong association between DRB1*04 and PV (Khan et al., 2015).

**Japan** A group with seventeen Japanese PV patients was also investigated and it was concluded that DRB1*0403, DRB1*0404, DRB1*0406 or DRB1*1401, DRB1*1405,

DRB1*1406 alleles were present, either one at a time or both simultaneously (Yamashina et al., 1998).

### 8.1.4. America

**Venezuela** The genetic susceptibility was also studied in Venezuelan patients, with a cohort of sixty six non-Jewish patients, in which forty nine were diagnosed with PV (50% of patients had mucosal involvement and the other 50% had cutaneous phenotype) and seventeen were diagnosed with PF. DRB1*0402 and DRB1*1401 were statistically significant to associate with PV and PF. Moreover, a haplotype analysis revealed that DRB1*0402 usually co-exist with DQB1*0302 and DRB1*1401 with DQB1*0503. These results demonstrate that DRB1*0402 is the most relevant responsible for pemphigus susceptibility in Venezuelan patients (Sáenz-Cantele et al., 2007).

**Brazil** A cohort with thirty wight EPF patients living in endemic areas was conducted to evaluate HLA genes. The variant HLA-DRB1*010 was found to be the main susceptibility factor to trigger the disease, and only two amino acids distinguish DRB1*0102 from DRB1*0101. The two amino acids seem to be responsible for the formation of a functional epitope, causing T cell recognition, and thus determining disease susceptibility (Moraes et al., 1991). Later, another study with one hundred and twenty eight EPF patients was conducted, and it was found that the more significant alleles among patients were HLA-DRB1*0101, HLA-DRB1*0102, HLA-DRB1*0103, HLA-DRB1*0404, HLA-DRB1*0406, HLA-DRB1*0410, HLA-DRB1*1406 and HLA-DRB1*1601 (Pavoni et al., 2003). On the other hand, a study with forty eight FS patients revealed that the haplotypes DR7,DQw2 and DR3,DQw2 seem to confer protection against EPF (Petzl-Erler and Santamaria, 1989). Also, concerning PV, the allele HLA-DRB1*0402 and HLA-DRB1*0804 and the HLA-DRB1*14 group have been implicated in PV susceptibility (Weber et al., 2011).
As aforementioned, little attention has been given to HLA class I in pemphigus. The HLA-B*16 was the only allele associated with PF susceptibility in Brazilians (Petzl-Erler and Santamaria, 1989). But very recently, a study with one hundred and sixty nine patients from São Paulo, Brazil, concluded that HLA-A*11 and HLA-A*33 allele groups confer susceptibility to PF, whereas HLA-A*02 is responsible for protection against PF. They also found an increased frequency of HLA-B*14 allele in PF patients, and a high frequency of the HLA-B*38 allele group in PV patients. Concerning the PV patients, they also found out that the allele group HLA-B*15 confer protection against the disease. For the first time it was described HLA-DRB1*14:01 alleles for susceptibility and HLA-DRB1*07:01 alleles for protection against PV disease. Moreover, they found the new genetic variant HLA-DQB1*0602 that is also associated with PV protection in this population. So, the HLA-DRB1*0102 and the HLA-DRB1*0402/HLA-DRB1*1401 alleles are the major etiologic fraction values in PF and PV, respectively. Until now, there were only a few reports linking the Brazilian population with HLA class I alleles in the disease's pathogenesis and this findings are truly important to better understand the value of these genes in the pemphigus process (Brochado et al., 2016).
The genetic predisposition in FS led researchers to conduct studies with Brazilian Mestizos and Xavante Indians that demonstrated particular HLA alleles. These specific alleles confer increased risk for the disease. The Terena Indians show some genetic

particularities. A cohort with twenty Terena Indians revealed that nineteen of them were positive for DRB1*0404, 1402 or 1406 which is concordant with Xavante findings. However, Mestizos had an association with DRB1*01. What is interesting in these alleles for all three populations is that all shared the same amino acid sequence at position 67-74 on the third hypervariable region of the DRB1 gene: LLEQRRAA. These findings suggest that inheritance of this sequence might be involved in FS susceptibility (Moraes et al., 1997).

### 8.2. BP Also Has Something To Do With Genetics

Although this relationship is somewhat tenuous, there are some studies looking for a concret answer. Recently, in 2015, a study with German population found out a link between mitochondrially encoded ATP synthase 8 gene (MT-ATP8) and BP. This could give a new insight about novel strategies to handle therapeutic approaches (Hirose et al., 2015).

Another study from 2015 investigated the possible link between CYP2D6 gene polymorphisms and BP cases. It seemed important to see if cytochrome 450 had an important role in BP pathogenesis. Seventy one BP patients were investigated and it was possible to identify the gene alleles: CYP2D6 (CYP2D6*1, CYP2D6*3, CYP2D6*4), of which CYP2D6*3 and CYP2D6*4 had a higher frequency than in controls. So this could mean that there is a relative risk for BP development four times higher in patients with these polymorphisms, especially with CYP2D6*3 presence (Rychlik-Sych et al., 2015).

### 8.3. Familial Cluster

Studies in families with pemphigus could provide further information of the genetic and environmental factors involved in pathogenesis. However, familial cases are barely found in the literature. The majority of the familial cases so far have been between mother and daughter and between siblings, so this constitutes the confirmed occurrence. The HLA typing studies demonstrated the same HLA alleles of HLA-DR4 (DRB1*04) and HLA-DQB1*03 in both cases (Eskiocak et al., 2016). The first Turkey case was reported between a brother and a sister, from Armenian origin. Both revealed histologic examination, clefts with acantholytic cells. Also there was an IgG and C3 deposition. Genetic analysis also had similarities, proving a genetic influence. Brother and sister presented localized lesions, essentially in exposed body areas (Gokdemir et al., 2006). Some of the cases found in literature are summarized in **Table 5** and as it is possible to confirm, there are not so many.

**Table 5 – Some familial pemphigus vulgaris cases found in the PubMed database. PF: pemphigus foliaceus, and PV: pemphigus vulgaris (adapted from Eskiocak et al., 2016).**

| Disease | Patients Relation | Localization | Author, Date |
|---|---|---|---|
| PV | Three brothers, two brothers and a brother and sister | Spain | Bordel-Gómez et al., 2006 |
| PV | Two sisters | Greece | Stavropoulos et al., 2001 |
| PV | Brother and sister | Turkey | Gokdemir et al., 2006 |
| PV | Brother and sister | Turkey | Kavak et al., 2005 |
| PV and PF | Daughter with PF and mother with PV, one sister with PF and other sister with PV | Japan | Yamamoto et al., 2011 |

### 8.4. Involvement of other genes

More recently it has been reported the participation of ST18 gene in PV, being up-regulated in the skin of PV patients. This event could be explained by the discovery of a PV-associated variant in the promoter region of the gene, variant responsible to increase gene transcription in a p53/p63 manner (Vodo et al., 2016). The ST18 gene is indicated to act as transcriptional regulator and is expressed in many normal tissues (Jandrig et al., 2004). These findings could lead us to a novel genetic pathway in order to better understand PV pathogenesis and potential treatment targets for this disease. Moreover, another study found a correlation between ST18-associated variants that might predispose to PV within some specific populations. This correlation was confirmed in Jews and Egyptians, where this gene was found overexpressed in patients' skin (Sarig et al., 2012).

Another finding with PV patients with Slovak origin confirmed associations between twentyn two single nucleotide polymorphisms (SNPs) in thirteen cytokine genes and PV. Unfortunately, only a weak association was found between TNF-α and IL-10 gene polymorphisms, which might give some contribution of genetic susceptibility to PV patients (Javor et al., 2010).

Capon et al. also demonstrated an association between PV patients and the Dsg3 gene, conferring susceptibility.Conducting a cohort with sixty two PV patients and one hundred an fifty four healthy controls from U.K. and another cohort with twenty eight PV patients and ninety eight healthy controls from northern India, they reported a significant association between PV and the DSG3*TCCTC haplotype, in the U.K. sample, and an association between the DSG3*TCCCC related haplotype and PV, in the India sample. Also, they found out that all British and all Indian patients with DSG3 risk haplotypes also carried at least one copy of a HLA allele. This demonstrated that the genetic variants of Dsg3 might be an additional predisposing factor to PV (Capon et al., 2006).

The non-classical HLA-E plays a role in autoimmunity, so the association with PV was investigated with fifty two Caucasian Ashknazi Jewish PV patients. HLA-E*0103X was found increased in these patients, providing the first reported data as a marker for genetic risk in PV patients (Bhanusali et al., 2013).

It was also studied the possibility of a linkage between a single nucleotide polymorphism (SNP) in the promoter region of TNF-α at position -308, affecting G to A transition, and pemphigus. The idea was to analyse the distribution of this SNP in North Indian cohort. Success was not achieved, since there was association with pemphigus risk in population at large. However, TNF-α could contribute to autoimmune phenomenon in pemphigus, being one of its multifactorial aetiology (Dar et al., 2016).

### 8.5. Concluding Remarks

All these data combined should be sufficient to clarify that genetic susceptibility does play an important role in these AIBD pathogenesis.

Also, the phenomenon of family aggregation should be better studied, so that one can definitively discern whether or not there really is a familial clustering that could de important for the patients' family and if this familial clustering could interfere with treatment, that is, if it could confer some kind of resistance. The occurrence of PV in first-degree relatives could indicate new paths for the importance of genetic predisposition.

Overall, nowadays, it is practically safe to say that genetics is a susceptibility factor.

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# Chapter IX

## Treatment

The main therapeutic backbone in pemphigus is the systemic corticosteroids that usually are combined with other immunosuppressants, such as azathioprine, mycophenolate mofetil or intravenous immunoglobulin (IVIg) (Daniel and Murrell, 2014). And although the immunosuppresants like azathioprine, mycophenolate mofetil, cyclophosphamide, cyclosporine, intravenous immunoglobulin (IVIg), and plasma exchange aren't enough for disease remission, they help in relapse decreasing it by 29% (Atzmony et al., 2015).
First of all, it is necessary to set some parameters before cortisteroid or immunosuppressive therapy. These are: complete blood count, creatinine, blood electrolytes, transaminases, gamma GT, alkaline phosphatase, total serum protein, albumin, fasting serum glucose, hepatitis B, C, HIV and chest x-ray. Moreover, there is also a set of optional analysis, such as: serum IgA deficiency, which is recommended to be ruled out before IVIg therapy; analysis of TPMT activity, before azathioprine therapy; optinal abdominal sonography, osteodensitometry, which is needed before glucocorticoid treatment; ocular examination; β HCG, to rule out pregnancy in women (Hertl et al., 2015).
Corticosteroids could be devastating to the patient's organism (Poetker and Reh, 2010). However, since they seem to be a crucial therapy for pemphigus, the side effects need to be well understood and controlled.

## 9.1 First-line Treatments

### 9.1.1. Corticosteroids

Since the discovery of prednisolone – and other corticosteroids – they have been widely used in pemphigus treatment (Fine, 1995). It is used as a first-line treatment, a systemic corticosteroid therapy, at 0.5 mg to 1.5 mg/kg/day. Usually, to control PV, a higher dose of prednisolone is required than to control PF. It is agreed that if PV is not controlled within 2 weeks, a higher dose can be used, up to 2 mg/kg, but this is an optional choice (Hertl et al., 2015).
Two case reports of females with PV - with forty three and sixty seve years old – attest that they responded well to oral prednisolone, in the treatment of systemic lesions. Also, when localized lesions appeared in both patients, this medication continued to be effective (Yoshifuku et al., 2016).
A French multicentre study compared topical therapy with 40g clobetasol propionate cream applied daily, to systemic treatment with 1mg/kg prednisolone, and reported that the topical glucocorticosteroid therapy had a better result, concerning disease control and mortality rate in active BP (Joly et al., 2002).

## 9.2. Second-line Treatments

### 9.2.1. Immunosuppresants

Additional immunosuppressive compounds, like azathioprine and mycophenolate mofetil, are used in pemphigus treatment (Almugairen et al, 2013; Hammers et al., 2013) in association with systemic corticosteroids (e.g 1.0-1.5 mg/Kg of prednisolone) (Hertl et al., 2015).

In **mycophenolate mofetil** administration, the usual dose is 2g/day, with the possibility to start with 500 mg daily and to raise the dose by 500 mg per week, until reaching the final dose of 2g/day (Hertl et al. 2015). A study with thirty one PV patients and eleven PF patients reported rates of remission in both diseases with mycophenolate therapy, 71% in the first group and 45% in the second one (Mimouni et al., 2003).

**Azathioprine (AZA)** is usually administrated at 1 to 3 mg/kg/day (Hertl et al., 2015). However, in the first week it's usually administrated at about 50 mg/day in order to detect idiosyncratic reactions (in that case, the medication is immediately stopped) (Ruocco et al., 2013). After the first week, the dose can be raised to desired dose. It's important to highlight that the activity of thiopurine methyl transferase (TPMT) needs to be monitored prior to azathioprine treatment (Meggitt et al., 2011; Hertl et al., 2015). Thiopurine methyl transferase has a major role in thiopurine drugs metabolism, like azathioprine (Fujita and Sasaki, 2007; Lee et al., 1995). Adults with high rates of TPMT activity can be treated with normal doses of azathioprine, up to 2.5 mg/kg/day. However, patients with intermediate or lower rates of TPMT activity should be more cautious in azathioprine intake, receiving a lower dose, up to 0.5 to 1.5 mg/kg/day or not be treated at all if the patient lack of TPMT activity (Hertl et al., 2015). Defects in the TPMT gene will eventually lead to enhanced bone marrow toxicity which could cause myelosuppression, anaemia, bleeding tendency, leukopenia and infection (Fujita and Sasaki, 2007; Lee et al., 1995). AZA also had some severe side effects, such as leukopenia, thrombocytopenia, anemia, pancytopenia and hepatotoxicity (Ruocco et al., 2013). It is noteworthy that drugs do have interactions with azathioprines, such as allopurinol, which are xanthine oxidase inhibitors (Meggitt et al., 2011). This drug component is administered, among others, in situations of inflammatory diseases (Pacher et al., 2006).

**Cyclophosphamide** is usually prescribed at 500 mg as intravenous (Zivanovic et al., 2010). Cyclophosphamide pulse therapy is thought to be safe and effective, but only in alternative cases of pemphigus, both PF and PV, of difficult control and response (Fernandes and Zubaty, 2005). Also, cyclophosphamide is effective in BP patients (Ruocco et al., 2013).

Azathioprine and cyclophosphamide revealed to be similarly effective in the pemphigus vulgaris treatment after about six months of therapy. The main difference is that azathioprine show a slower onset of action, with improvements seen in six months, whereas cyclophosphamide show a faster onset of action with improvements seen in three months (Sardana et al., 2016). The most alarming information is the reports of patients dying when receiving cyclophosphamide, concerning cardiotoxicity (Sardana et al., 2016). This adjuvant immunosuppressive compound is administered only if patients didn't achieve remission with main corticosteroid therapy and AZA or mycophenolate mofetil, suffer from severe side effects with previous therapy or if the patient have rapid disease progression (Meurer, 2012).

In BP patients, **leflunomide** is also used as a therapy. Leflunomide acts more like an anti-inflammatory than an immunosuppressive and it is more used in BP than in other pemphigus. Usually, 20 mg are administered, combined with 20 mg of prednisone, every day for 5 weeks. After 5 weeks of therapy, the corticosteroids begin to taper and leflunomide is reduced to 10 mg per day (Ruocco et al., 2013). There are reports of patients that have only responded to high prednisone doses, but have respondeded well to leflunomide (Sehgal and Verma, 2013).

There is a compound that seems to be more efficient in treating BP patients than pemphigus ones – **Methotrexate (MTX)**, a drug used to treat cancer and autoimmune diseases (Culton and Diaz, 2012). MTX has a lower incidence of infections in patients when compared to those treated with cyclophosphamide, mycophenolate mofetil or AZA. MTX is usually used twice a week in oral doses with 5 to 25 mg, and it seems to work very well a as systemic monotherapy, being safe and effective as a BP therapy. It is indicated for patients with contraindication for 'systemic corticosteroids', for the elderly with multiple clinical problems and for patients with a mild or limited form of the disease (Gürcan and Ahmed, 2009). However, MTX also have its side effects, including pancytopenia and hepatotoxicity, so patients with renal disorders and without any acid folic supplementation are at a great risk (Culton and Diaz, 2012).

**Dapsone**, a glucocorticoid-sparing agent was reported to be effective in the maintenance-phase of PV (Baum et al., 2016[b]; Werth et al., 2008). In elven patients treated with dapsone, eight were successfully treated, presenting an efficiency of about 73% (Werth et al., 2008). Dapsone is a great therapy in the maintenance phase of PV disease, since it reduces the steroid dependence in patients (Heaphy et al., 2005). These immunosuppressant compounds are also used in BP treatment, as corticosteroid-sparing agents, and the complete remission of the disease can be achieved (Tirado-Sánchez et al., 2012). In BP treatment, 100 mg per day can administered, with 60 mg of prednisone. However, dapsone dose can be increased (50 mg weekly to a maximum of 200 mg per day) if there is no improvement. Adverse effects can be reversible and depend on the dose (Ruocco et al., 2013). There is a case report of a fifty year old woman, diagnosed with PV that evolved to a drug-induced hepatitis after dapsone use (Quaresma et al., 2015). So, despite dapsone being a relatively safe drug, a laboratorial monitoring is important in order to identify side-effects during its use.

**Table 6 – Few possible combinations for treatment of autoimmune blistering skin diseases (adapted from Schmidt and Zillikens, 2011).**

| Disease | Treatment |
|---|---|
| Pemphigus Vulgaris<br>Pemphigus Foliaceus | Prednisolone (1.0-2.0 mg/kg/d[*1])[*2] + Azathioprine or Mycophenolate mofetil or Cyclophosphamide |
| Bullous pemphigoid | Clobetasone propionate 0.05% cream /10-30 g/d)[*3] + Dapsone or Doxycycline or Azathioprine or Methotrexate |

***1: initial dosage, can be lowered depending on the clinical response.**
***2: alternatively.**
***3: it was shown that clobetasone propionate 0.05% cream (10–30 g/d) in a tapering dose is just as effective as prednisolone (0.5 mg/kg/d); with fewer side effects.**

### 9.3. Third-line Treatments

#### 9.3.1. Rituximab - anti-CD20 antibody

Nowadays, rituximab is used as a third-line treatment (Hertl et al., 2015); yet, some studies to make this medication as a first-line treatment are underway. It can be combined

with intravenous immunoglobulin (IVIg) or immunoadsorption. Rituximab's efficacy derives from factors such as CD20 being expressed in high levels on B cells and not internalizing or sheding from plasma membrane after mAb treatment (Glennie et al., 2007). Anti-CD20 antibody is used to cause the depletion of $CD20^+$ peripheral B cells, which last for at least half a year. After that, the B cells repertoire reconstitution, with naïve and transitional B cells, will derive from the stem cell pool (Colliou et al., 2013). This therapy seems to be effective in both PV and PF treatment (Cianchini et al., 2007) and is also effective and well tolerated in BP patients (Kasperkiewicz et al., 2011). Nontheless, the high costs still limit the use of this biologic compound to patients' treatment resistance or life threatening disease (Cianchini et al., 2007).

This monoclonal antibody is usually administrated 2 x 1g intravenous, with two weeks apart, or 4 x 375 $mg/m^2$, each with one week apart (*in* Ahmed et al., 2006; Hertl et al., 2008, 2015; Joly et al., 2007; Kasperkiewicz et al., 2012). And since rituximab can be administrated along with IVIg or immnunoadsorption, it is important to know their recommendations. IVIg can be prescribed at 2g/kg/month and the immunoadsorption is performed in two cycles in four consecutive days, with 4 weeks apart (Behzad et al., 2012; Hertl et al., 2015; Kasperkiewicz et al., 2012; Zillikens et al., 2007). The IVIg therapy also provides an alternative for BP patients that resist to corticosteroid therapy (Amagai et al., 2016). Side effects with IVIg are usually lower than 1%, and that includes headache, back pain, chills, flushing, fever and hypertension, all of them related to the rate of infusion and not to the dose (Lo Schiavo et al., 2010). Transfusions may also lead to moderate effects like fever and chills, during first transfusion. Thirty to one hundred and twenty minutes after infusion, patients can possible feel nausea, pruritus, angioedema, asthenia, hypotension, headache, bronchospasm, vomiting, throat irritation and dizziness, which can all be solved if the transfusion slows down or gets interrupted. Still, some aggravated reactions can occur with a fatal outcome. Hepatitis B reactivation with fulminant outcome, cardiac arrhythmias, renal toxicity, bowel obstruction and perforation, among others, has been described in BP patients (Lo Schiavo et al., 2010).

Additionally, IVIg) helps in decreasing levels of pathogenic anti-Dsg antibodies (Danieli and Schoenfeld, 2014). This decrease happens so quickly that the explanation for the mode of action could be explained by induction of general increase in the antibodies' catabolism, which includes the pathogenic ones (Czernik et al., 2008). It was reported in 2005 that the keratinocyte detachment and death in PV from a sort of synergistic action of apoptosis effectors and the IVIg therapeutic activity is, in part, mediated by the stimulation of events of anti-apoptosis (Arredondo et al., 2005).

Heelan et al. reported that the median time to relapse, after the first treatment cycle of rituximab, was 15 months, in which all patients demonstrated a notable improvement (Heelan et al., 2014).

Patients treated with rituximab earlier in disease may have higher chances of having better outcomes, maybe due to the ability of destroying all nontolerant anti-Dsg B cell clones (Colliou et al., 2013; Lunardon et al., 2012).

Eming et al. believed that, presumably, the drug has the ability to downregulate Dsg3-specific $CD4^+$ Th cells (Eming et al., 2008). This could open new perspectives of drug action mechanisms for this drug (Zambruno and Borradori, 2008).

Rituximab proved to result in disease control by decreasing B cells, precursors of short-lived plasma cells that can produce pathogenic antibodies. Also it was possible to

determine that a lower peak serum of BAFF levels, right before B cell recovery, seems to predict an early relapse of skin blistering, so this could be an indicator for earlier treatment with rituximab (Hall III et al., 2013). Also, the use of rituximab enables a lower initial dose of oral prednisolone, reducing total dose. Adverse effects of this therapy were rarely reported (Sharma et al., 2016).

A study with rheumatoid arthritis (RA) patients administered with rituximab showed that these suffered a depletion of all B cell populations. However, after a mean of 8 months after treatment B cell repopulation occurred and this repopulation mainly originated naïve mature and immature B cells. So, this treatment indeed induces a depletion of all peripheral blood B cell populations, at least in RA patients (Leandro et al., 2006). And the same happens in pemphigus patients (Mouquet et al., 2008). However, a study with systemic lupus erythematous (SLE) treated with rituximab demonstrated that the reconstitution of B cell, after depletion, may be functionally immature, where mostly are transitional B cells. These results demonstrate the peripheral blood memory reconstitution and that happens because the reconstitution is dominated by immature transitional B cells (Anolik et al., 2007). More studies about B cell depletion after this kind of treatment is needed to enhance the ability to rationally tailor the therapy in patients with autoimmune diseases.

Rituximab has been demonstrated to be a promising first line therapy for pemphigus, and so have other biologic medications, like veltuzumab, obinutuzumab, ofatumumab, ocaratuzumab and belimumab (Huang et al., 2016).

### 9.3.2. Depletion of autoantibodies: an adjuvant strategy

Therapy based on plasma exchange (TPE), also known as plasmapheresis, has the potential to remove harmful large molecular-weight substances, such as autoantibodies, whereas, immunoadsorption (IAS) is used as a blood-purification technique that selectively removes immunoglobulins (Ig) from separated plasma through high-affinity adsorbers (Honoré et al., 2014).

Although plasmapheresis and immunoadsorption show high efficacy to quickly remove the excess of pathogenic antibodies from the serum, they are still used as adjuvants. They have the necessity to act combined with systemic immunosuppression in order to prevent the rebound of newly synthesized antibodies (Turner et al., 2000; Kasperkiewicz et al., 2012[b]). This technique involves the removal of large amounts of plasma during several days and it is done during hospitalization. However, plasmapheresis has a high cost and it's associated with potential morbidity. For this reason, it should not be recommended as a routine therapy for BP (Ruocco et al., 2013[b]). A case report of a sixty three year old man with refractory BP that went through double filtration plasmapheresis shows that, in fact, the skin lesions did improve, however, factor XIII and fibrinogen levels decreased and deficiency of these factors can possibly cause lethal bleeding due lack of coagulation capacity. So plasmapheresis was switched by selective plasma exchange, an improved plasmapheresis that uses membrane plasma separator with smaller than ordinary pores. This new therapy also demonstrated good results without decrease factor XIII and fibrinogen levels. This therapy can represent an alternative for classic plasmapheresis, reducing pathogenic antibodies, as intended, and without decreasing levels factor XIII and fibrinogen, which take a long time to recover (Nasu et al., 2016).

### 9.3.3. Gold Salts

The introduction of gold for pemphigus treatment, intramuscularly, has generated positive results over time (Iranzo et al., 2007; Lange et al., 2007). The intramuscular injections are made of gold sodium thiomalate or autothioglucose and are administered once a week, in single doses with 50 mg to 1g, whereas Auranofin is an oral preparation, administered 3 mg twice every day. Auranofin seems to be less toxic than the injections, but also less effective (Ruocco et al., 2013). Nevertheless, this therapy had serious toxic effects in about 42% of the patients (Pandya and Dyke, 1998).

The use of this therapy is not recommended for pemphigus patients, being banned with the use of other immunosuppressive agents (Hammers et al., 2013; Ruocco et al., 2013).

### 9.3.4. Tetracyclines and Nicotinamide

The use of these two agents could be helpful when we have mild cases of pemphigus disease. It is administered as an association of 2g per day of oral tetracycline or 0.5 to 0.20g per day of minocycline, together with 1.5g per day of nicotinamide (see Harman et al., 2003 and included references). Moreover, concomitantly tetracycline administration as a combinatory therapy will help the dosage of immunosuppressant be tapered more rapidly (Sapadin and Fleischmajer, 2006). Tetracycline also demonstrated to be a useful alternative in BP cases (Walsh et al., 2005).

## 9.4. Supportive treatment: additional options

Besides all medication mentioned above, there are also some additional measures available which may complement the main treatment.

Sometimes, intralesional injections of cortisteroids are administered, such as triamcinolone acetonide, which could play an important role in isolating lesions of oral mucosa, lips and skin, for instance. It is also possible to apply potent topical cortisteroids, like clobetasol propionate, or calcineurin inhibitors, and oral corticosteroids, like triamcinolone acetonide gel, directly in the lesion, in oropharyngeal erosions bcombined with systemic therapy (Cohen et al., 2006; Hertl et al., 2015; Iraji and Banan, 2010). Another extra recommendation is that, in case of erosive lesions, patients take analgesics, such as paracetamol, metamizol and opioids, and even gels containing local anaesthetics can be used for application. A dental care is also absolutely required (Hertl et al., 2015).

The bullae that didn't suffered erosion and remained intact should be punctured and in the wound sprays or lotions containing corticosteroids and antibiotics like gentamicin should be applied. Sometimes, baths with aseptic solutions like potassium permanganate are also necessary, in concentration 1:10 000, or even chlorohexidine. In cases with oral lesions that resist to systemic therapy, it's also used topical corticosteroids, like clobetasol propionate. Another option is the intralesional injections with triamcinolone acetonide (10 mg/mL). Sometimes, these oral lesions also cause discomfort during food intake, so it's also recommended the application of anaesthetic nebulizers like benzocaine (Ruocco et al., 2013). Nowadays there are some innovative alternatives that try for the reepithelization of the lesion caused by the disease. We're talking about epidermal growth

factor (Tabrizi et al., 2007), nicotinamide gel (Iraji and Banan, 2010) and pimecrolimus 1% (Tyros et al., 2013).

In the case of patients in long-course therapy with cortisteroids, it is also highly recommended that they are screened for osteoporosis and, if necessary, manage prophylaxis. Management of glucocortisteroids for the smallest dose for the shortest time as possible is also taken into account, in order to minimize osteoporosis risk. To prevent further damages, patients also need to take vitamin D and calcium supplements, and in case of patients at risk, such as post-menopausal women, treatment with bisphosphonates is recommended. Patients should also be careful and take regular ophthalmologic evaluation. They can also take oral topical antifungals for prophylaxis of oro-intestinal conditions. A psychological support should also be given to patients, to try to alleviate the condition and to give them tools to overcome their situation. Moreover, physiotherapy sometimes is necessary in patients with prolonged cortisteroid therapy (Hertl et al., 2015).

### 9.5. Future perspectives

Along with all the treatment options available, new studies have been made hoping to be able to minimize the damage of steroids therapy.

**Omalizumab**, monoclonal antibody (mAb), a recombinant humanized mAb anti-IgE (Balakisski et al., 2016), has already been proposed as a viable alternative for BP patients, for instance. Still, it needs further studies to evaluate if it can be used as a monotherapy or as an adjuvant agent (Fairley et al., 2009).

Also, **ustekinumab**, a mAb that prevents interaction of IL-23 and IL-12 with their respective receptors, so will block the Th1 and Th17 differentiation. As aforementioned, Th17 initiates the inflammatory cascade in BP, so this new approach can represent a new hope (Lo Schiavo et al., 2013). In a typical case of BP associated with psoriasis that relapsed, ustekinumab demonstrated good results controlling the disease (Loget et al., 2016). Treatment with IL-17 inhibitors is already used in patients with psoriasis, a disease that, like BP, is caused by an exacerbated production of inflammatory cytokines. Two more anti-IL-17 are under clinical trials, since February 2015 – brodalumab and ixekizumab. These biological agents have been demonstrated efficient and safe. These drugs can also be useful in PV and BP treatment (Wasilewska et al., 2016).

Likewise, the chimeric antigen receptor (CAR) was tested to be used as PV therapy. Human T cells were engineered to express chimeric autoantibody receptor (CAAR). Though still under study, this therapy could represent a new hope in PV therapy (Ellebrecht et al., 2016).

Because of the controversy caused by the standard pemphigus treatment, innovative strategies have been developed. As aforementioned, a tandem peptide could target the *trans* adhesive interfaces of desmogleins to crosslink them and stabilize adhesion, inhibiting skin blistering and activation of p38MAPK pathway, through PV-IgG (Spindler et al., 2013).

Another novel tool could be the manipulation of plakophilin-1. Tucker et al. demonstrated the key role of manipulation of plakophilin-1 (PKP-1) expression, which is an intracellular armadillo protein, capable of linking desmosomal cadherins to keratin intermediate filaments of keratinocytes. It was reported that when they enhanced the PKP-1

expression, this protein could protect keratinocytes from pemphigus vulgaris IgG-induced loss of cell-to-cell adhesion. Moreover, PKP-1 seems to cluster Dsg3 with the desmosomal plaque protein desmoplakin, and transform 'desmosome adhesion from a calcium-dependent to calcium-independent and hyper-adhesive state' (Tucker et al., 2014). Similar results could be obtained inserting a point mutation, S2849G, into desmoplakin, which causes an inhibition of both Dsg3 depletion from cell surface and keratin filament retraction, caused by PV-IgG. This inhibition occurs due to preventing protein kinase C-dependent phosphorylation of desmoplakins, at that specific site. We can be in the presence of a new pharmacological tool in pemphigus, since protein kinase C inhibitor Bim-X compound seems to have the same inhibitor effect (Dehner et al., 2014).
The inhibition of various signalling pathways that have implication in pemphigus antibody-induced acantholysis could represent a key role in a new therapeutic approach (Mao et al., 2014).
Moreover, the future of ABD treatment might undergo pharmacological protection of mitochondria and/or compensation of the injured mitochondrial functions. This can became a new approach to develop personalized non-hormonal therapies to treat ABD (Kalantari-Dehaghi et al., 2013[b]). The use of engineered Fabs also appears to be a great strategy for ABD treatment, since it can block pathogenic epitopes leading to more specific therapies (Wang et al., 2010).
The effect of pilocarpine has also been under perspective for PV treatment. This M1 muscarinic acetylcholine receptor agonist was reported to improve pemphigus acantholysis, inhibiting PKC-dependent serine phosphorylation of β-catenins and tyrosine phosphorylation of p120-catenin. This study also reinforces the major role of the acetylcholine (Ach) signaling center line in regulation and coordination of different events that assemble and disassemble the intercellular junctions of keratinocytes (Chernyavsky et al., 2008).
There is another therapy that targets the complement cascade (Ricklin and Lambris, 2007) that could be applied in BP cases, however, has not yet been properly evaluated in these cases (Hammers and Stanley, 2016).

### 9.6. Concluding Remarks

It is known that our knowledge about pemphigus has suffered a tremendous progress over the years, however there are many obstacles when analysing outcomes of treatments. So, consensus statements are needed for better clinical evaluations of pemphigus' patients (Murrell et al., 2008).
Frequently, pemphigus is a fatal skin disease, fact that was studied by Ahmed and Moy. They accessed the data from thirteen patients' autopsies that died from this disease, at the UCLA Hospital, between 1965 and 1980. It was concluded that infection was the most frequent cause of death, being septicaemia the most important one, being present in 9/13 of the cases (69.2%). *Staphylococcus aureus* was found to be the most important organism. Moreover, nine patients also had pneumonia. These patients were on long-term high doses of cortisteroids, which could also mask the symptoms of inflammation (Ahmed and Moy, 1982).
The severe harmful effects and complications that the corticosteroid hormone (CH) therapy entails do not always produce a positive balance between treating and not

treating the patient. It is very clear that there is an urgent need for better and novel therapies that could possibly help in keratinocyte adhesion or that maybe could antagonize pemphigus autoantibodies.

## 9.2 Final Disclaim

This chapter bases itself on the information conveyed by the indicated references, information contained therein and interpretation made by the author of the same, and should not be used outside the context of this work. The author disclaims any responsibility for the inappropriate use of the information in particular concerning the therapy. The aforementioned drugs may have one or more side effects, and their use must include appropriate precautions for each active principle, their association with them, and should be prescribed by experienced health professionals and through appropriate monitoring.

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# Chapter X

**Epidemiology – what's happening around the globe?**

Bullous pemphigoid, pemphigus vulgaris and pemphigus foliaceus seems to be scattered all over the world. In Africa predominates the PF, and in some parts of Asia and America. In the other hand, BP and PV appear to be widespread in Europe and Asia.
For instance, in North Africa and Middle East countries, represented in **Figure 20**, the predominant subtype may varies: PV is more common in Egypt, Sudan, Morocco, Syria, Kuwait, Saudi Arabia and Yemen; and PF is predominant in Libya and endemic in Tunisia, and within Morocco is more common in Marrakech (Saleh, 2015).

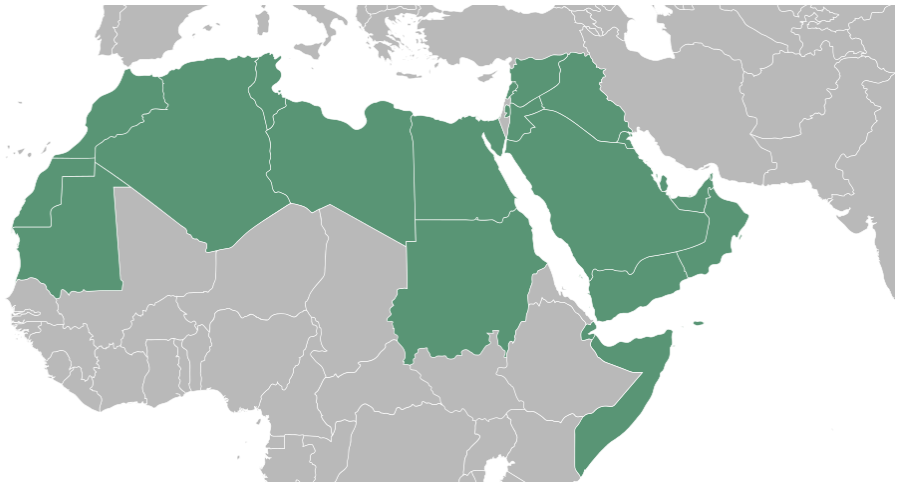


**Figure 20 – Countries of North Africa and Middle east with reported PV, PF and/or BP.**

Over the years, researchers and clinicians have been facing difficulties in establishing mortality rates of patients with pemphigus, around the globe.
When we talk about pemphigus, a mystery remains concerning the rates, since the mortality decreased from 75% (pre-corticosteroids), in the 1950s, to 30% (Bystryn and Steinman, 1996). Many studies reported mortality rates between 4.8% and 25.9%, mainly due complications of long-term immunosuppression (Ahmed and Moy, 1982; Uzun et al, 2006). For instance, in the 50's, if a pemphigus patient was left without treatment, the next 2 years the mortality rate was 50% and at the end of 5 years the mortality rate raised to 100% (Mimouni et al., 2008). Confirming the mortality rates, a study with 138 PV patients and 551 matched controls, conducted in 2008, concluded that in one year the mortality of these patients was 12%, which was three times higher than controls (Langan et al., 2008).
It also important to differentiate the mortality of patients admitted with a primary diagnosis of pemphigus and the ones admitted with a secondary diagnosis of pemphigus. So, Hsu et al., 2016 conducted a study in the U.S.A., using the International Classification of Diseases, Injuries and Causes of Death (ICD-9) codes, regarding the inpatient mortality in pemphigus. They analysed a cohort of 87 million admissions, between 2002 and 2012, which represented 20% of US Hospital admissions. These analyses led to the conclusion that 1.6% of the admissions were primarly due to pemphigus diagnosis. For a secondary admission it was about 3.2%. And this value was higher than controls. In these last cases, the admissions were mainly due to comorbidities, such as infections (Hsu et al., 2016).
BP has been reported to have 20% to 40% mortality rate in the first year after the diagnosis (Hübner et al., 2016).

The incidence of pemphigus depends on what country or region are analysed, ranging from 0.5 to 32 per million per year. Differences are also found if we compare the incidence between gender, age and, in several situations, ethnicity (see review in, Alpsoy et al., 2015). The annual incidence of pemphigus, in Europe, has been ranging between 0.5 and 8 per million (Alpsoy et al., 2015), knowing that the higher incidence refers to the Mediterranean Basin (Meyer and Misery, 2010) whereas BP has a incidence ranging between 0.1 and 42.8/million per year in European countries (review *in*, Alpsoy et al., 2015), as its possible to see summarized in **Table 7**.

**Table 7 - Incidence of PV, PF and BP.**

| Continent | Country | Cohort | Pemphigus | Sex Gender | Mean age of onset (years old) | Incidence PV, PF / BP (per million) | Reference |
|---|---|---|---|---|---|---|---|
| Africa | Mali | | 25 PF / 4 PF | 80% women | 46.7 | 2.9 | Mahé et al. 1996 |
| | Tunisia | 23 pemphigus | 20 PF | Not specified | | 4 | Morini et al. 1993 |
| | Tunisia | 174 ABD | 53% pemphigus / 24% BP | Not specified | 50 for pemphigus / 68.6 for BP | 8.62 for pemphigus and 3.84 for BP | Zaraa et al. 2011 |
| | Tunisia | | 61% PF | | 36.7 | 6.7 | Bastuji-Garin et al. 1995 |
| Europe | Turkey | 148 Pemphigus | 123 PV 13 PF | | 43 | 2.4 | Uzun et al., 2006 |
| | Serbia | 51 Pemphigus | 72.55% PV and 11.76% PF | | 55.6 | 6.6 and 0.85 / 100 000 over age of 20 | Golusin et al., 2005 |
| | Greece | 129 PV | PV | 41 men / 88 women | 59.8 | 8 | Michailidou et al., 2007 |
| | Italy (Liguria) | 32 pemphigus | | 13 men / 19 women | | 10 | Cozzani et al., 2001 |
| | France | | 73% PV | | 52 | 1.7 | Bastuji-Garin et al. 1995 |
| | France | 502 BP | BP | | | 21.7 | Joley et al., 2012 |
| | Switzerland | 168 pemphigus and BP | | 73 men / 95 women | P 62.3 / BP 77.2 | 0.6 for PV and PF / 12.1 for BP | Marazza et al., 2009 |
| | Germany | Pemphigus / BP | | | | 94.8 for PV / 10.0 for PF / 259.3 for BP | Künber et al., 2016 |
| | Germany | 27 BP | | | 62 | 0.5 | Bertram et al., 2009 |
| | UK | 869 BP 138 PV | BP and PV | 61% women in BP and 91% women in PV | 71 | 6.8 for PV / 42.8 for BP | Langan et al., 2008 |
| | Scotland | 83 BP | | | 79.2 | 14 | Gudi et al., 2005 |
| | Denmark | | | | | 60 pemphigus / 120 for pemphigoid | Eaton et al., 2010 |
| Asia | Israel | 180 | 159 PV and | 66 men | 54.7 | 7.2 (Jewish | Kridin et al., |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | 20 PF | and 114 women | | 7.5; 3.6 Arabs) | 2016 |
| | Israel | 290 | PV | | 49.7 | | Baum et al., 2016 |
| | Iran | 1209 | PV | | 42 | 10 (16, Tehran) | Chams-Davatchi et al., 2005 |
| | Iran (Shiraz) | 221 | 87.7% PV and 9% PF | | 38 | 6.7 | Salmanpour et al., 2006 |
| | Iran (Isfahan) | 188 PV | PV | 72 men 116 women | 41.1 | 50 | Asilian et al., 2006 |
| | Iran (Yazd) | 89 Pemphigus | | 44.9% men and 55.1% women | 44.9 | 9.8 | Noorbala et al., 2012 |
| | Iran | 122 BP | | 35.2% males and 64.8% females | 65 | | Esmaili et al., 2012 |
| | India | 41 Pemphigus | 32 PV 8 PF | | | | Chowdhury et al., 2016 |
| | Thailand | 58 | BP | | 69.3 | | Kulthanan et al., 2011 |
| | Taiwan | 853 Pemphigus | | 42.9% men and 57.1% women | 52.5 | 4.7 | Huang et al., 2012 |
| America | Brazil | 20 | 12 PV and 8 PF | 66.7% women in PV and 75% men in PF | | | Pires et al., 2014 |
| | Brazil (endemic Region) | 266 | 163 PV and 103 PF | | 32.1 PV / 41.5 PF | | Gonçalves et al., 2011 |
| | Brazil (Amerindian) | 1351 | | | | 40.5 | Hans-Filho et al., 1996 |
| | Colombia | 130 | PF (FS) | 95.4% men | 50 | | Abrèu-Velez et al., 2003 |
| | USA (Connecticut) | Pemphigus | | | | 4.2 (32, in Jews) | Simon et al., 1980 |

## 10.1. Africa

### a. Mali

A study made in Bamako, Mali, reported that pemphigus in this specific area have a distinctive pattern, with an annual incidence of 0.29 cases per 100 000 inhabitants, more prevalent in women (80%) with a mean age of onset of 46.7 years; and a ethnic group named Fulani (33%). The cohort had 25 PF and 4 PV cases. In resemblance to what have been reported in Tunisia, pemphigus in Mali too differ from what happens in North America and Europe, and in Brazil (Mahé et al., 1996).

**b. Tunisia**

A cohort with 23 Tunisian pemphigus patients was assessed to perform a prospective study about PF occurrence in Tunisia. Of the 23 cases, 20 were PF mainly in young women. The incidence was estimated to be 4 new cases per million per year, higher than in European or North American, however, lower than most severely affected areas in Brazil. One of the characteristics that differentiate Tunisian pemphigus from PF observed in Europe, North America and Brazilian FS is the lack of familial cases and the major incidence in women (Morini et al., 1993).

Zaraa et al. performed a retrospective study between 1997 and 2007, gathering a cohort with 174 patients with ABD, where pemphigus was the most common with a mean age of onset of 50 years old, where 53% represent only the pemphigus cases (61% PV and 36% PF (Tunisian pemphigus)). Also, BP was the second most frequent with 41 patients suffering with it, with a mean age of onset of 68.6 years old. It was reported that pemphigus has an incidence rate of 8.62 new cases per year and BP 3.84 new cases per year. It was also reported that PF has a higher prevalence than in Western Europe (Zaraa et al., 2011).

Moreover, in previous studies, Bastuji-Garin et al. reported the high incidence of PF in Tunisia, when comparing with the incidence rates of pemphigus in a large area of France, which revealed an incidence rate of 1.7 cases per million, per year. PV represented 73% of all cases. Tunisia revealed an incidence of 6.7 cases per million per year. PF was more frequent, with 61% of the cases, and much more prevalent in young women aged 25 to 34 years old, with an incidence rate of 15.5 cases per million per year. These data, once again, demonstrates the unusual pattern of pemphigus in Tunisia, and patients were not related (Bastuji-Garin et al., 1995).

## 10.2. Europe

**a. Turkey**

In Mediterranean region of Turkey, Adana and Antalya, a prospective study was made, from 1998 to 2004, comprising a cohort with 148 pemphigus patients. From all the patients, 123 were diagnosed with PV and 13 with PF. The incidence was estimated to be 0.24/100 000 cases per year with a female predominance and a mean age of onset of 43 years. Also, in 82% of all PV patients the disease initiated as oral ulcers. This Mediterranean region has a relatively high incidence of pemphigus when compared with other countries (Uzun et al., 2006).

**b. Serbia**

A study conducted in South Bačka, Vojvodina, evaluated the incidence of pemphigus in this area. A 13 year period produced a total of 51 new pemphigus cases, with a peak in 1996, resulting an incidence of 0.66 cases per 100 000 inhabitants and 0.85 per 100 000 inhabitants in those over age of 20 years. Of all cases, 72.55% were PV and 11.76% were PF. The mean age on onset was estimated in 55.6 years, and a slight increase of women prevalence. Moreover, the autors did not found any significant seasonal influence (Golusin et al., 2005).

**c. Greece**

A study from 2007 reported an average incidence of eight new PV patients per year (0.8/100 000 inhabitants), in the northern Greece population, and a mortility rate was 2.3%. This study was made with 129 PV patients, 41 males and 88 females. It was possible to infer that the disease occurred most frequently in the sixth decade of patients' life. Also, the mean age of onset in males ranged 30 to 88 years and in females ranged 34 to 80 years (Michailidou et al., 2007).

**d. Italy**

A two-year study in Liguria intended to demonstrate the incidence of BP in this region. Liguria is a coastal region of north-western Italy and has about 1.7 million inhabitants. The 32 cases collected (13 males and 19 females) over the period of the study corroborates an average incidence of 1/100 000 cases per year, as proven previously (Cozzani et al., 2001).

**e. Switzerland**

A major study was done in Switzerland in 2009, encompassing January 2001 to December 2002. A cohort with 168 patients, 73 men and 95 women, with BP and pemphigus were collected. The results showed that BP had a mean incidence of 12.1 new cases per million per year and this incidence increases after the age of 70 years old. When we standardize the incidence in Europe, the incidence seems to be lower, with 6.8 new cases per million per year. In the other hand, the incidence of PV and PF combined is lower, 0.6 new cases per million per year. This study covered the entire country, so it is possible to stablish two things: first, if compared with PV and PF, BP is the most frequent autoimmune bullous disease and, second, this incidence have nothing to do with ethnicities, since it is not specified, but have to do with the advanced age of Swiss population (Marazza et al., 2009).

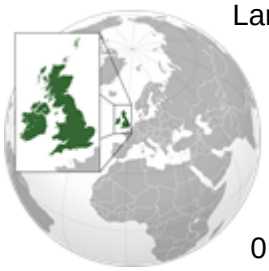
### f. France

A prospective study comprising three French regions with a total of 3.858 million inhabitants investigated the BP incidence. From 2000 to 2005, a cohort of 502 incident BP patients was identified. Overall, the incidence was estimated to be 21.7 cases per million per year, which is three times higher than the incidence in 1985. When they look close to population with 70 years or more, the incidence increases to 162 cases per million per year. In the first year, after diagnosis, the survival rate is estimated to be 62%. Moreover, the risk of death in BP patients is six times higher than in general population (Joly et al., 2012).

### g. Germany

In 2014 it was performed a study to evaluate the incidence of autoimmune bullous diseases, using data from a major Germany health insurance company. They concluded that in 80 925 million inhabitants in Germany, 40 400 suffer from pemphigus and pemphigoid diseases. Being BP the one with the major rate of incidence, with 259.3 patients per million. PV have an incidence of 94.8 patients per million and, PF have 10.0 patients per million. So, in this study, BP was determined to be 2.5 fold higher, when compared with PV and PF (Hübner et al., 2016).
Another study from 2009 reported the incidence of BP in Lower Franconia (the Franconian lands belong to Bavaria, and are located in the centre of Germany) with 27 BP patients diagnosed make this disease, once again the most prevalent among the group of the ABD, with 13.4 new cases, per million, per year (Bertram et al., 2009).

### h. United Kingdom



Langan et al., 2008, reported the incidence and mortality in the UK, with a an cohort with 869 BP patients and 138 PV patients, with a mean age of onset of 80 years in BP cases and for PV patients was 71 years. Both BP and PV were more prevalent in women, 61% of all BP patients were females and 91% of PV was female.
The incidence of BP was 4.3/100 000 and PV had a frequence of 0.7/100 000 person years. It was also seen that the BP incidence has been increasing over time of an average of 17% a year, and so PV has been increasing too, abou 11% per year. BP patients also had twice probability of death than controls and PV patients had three times more probability of death, also compared with controls (Langan et al., 2008).
The Grampian Region, northeast Scotlant, was investigated to assess causes of mortality of BP patients, with a cohort of 83 patients, collected between 1991 and 2001. The annual incidence was estimated to be 14 cases per million per year. It was also observed a rise in the incidence in the elderly, and 48% of patients died whithin 2 years of diagnosis, in the majority from respiratory disorders (Gudi et al., 2005).

### i. Denmark

A study from 2006 reported that the incidence of pemphigus and pemphigoid diseases were 60 patients per million and 120 patients per million, respectively. This study took into account that the entire population of 5 506 574 persons in Denmark on October 31, 2006 (Eaton et al., 2010).

## 10.3. Asia

### a. Israel

A very recent retrospective study estimated the incidence of pemphigus in Israel. The main difference between this study and the others e that here the authors also investigated the differences between two major ethnic populations, in two Israeli regions: Haifa, with 875 000 inhabitants, and northern districts, with 691 000, according to 2008 census. During a 16 year period, a cohort of 180 pemphigus patients was gathered, in which 159 were PV and 20 were PF; and 66 were men and 114 were women. The mean age of diagnosis was 54.7 years. It is the origin of the patients that gives us a new insight of epidemiologic Israeli studies, 59.4% were Ashkenazi Jews, 22.2% were Sephardic Jews and 17.2% were Arabs. So, the overall incidence was determined to be 7.2 cases per million per year, from 2000 to 2015, in northern Israel. The incidence in the Jewish sub-group decreased from 11 cases per million per year, in 2000 to 2005 to 9.3 cases per million per year in 2006 to 2010 and again decreased to 7.5 cases per million per year in 2011 to 2015. The contrary happened among the Arab sub-group, where the incidence slightly increased from 3.1 in 2000 to 2005 to 3.6 in 2011 to 2015 (Kridin et al., 2016).

Another retrospective study with data from a medical center in Israel, from 1980 to 2009, Jews, intended to investigate epidemiology in Jews, Ashkenazi and non-Ashkenazi. A cohort with 290 PV patients revealed that the mean age of onset was 49.7 years and, again, a female prevalence. The ratio of Ashkenazi and non-Ashkenazi, in Jews patients, was 1.23:1.00 (Baum et al., 2016).

### b. Iran

A study reported that the most prevalent form of pemphigus is PV, with an incidence of 1.0/100 000 per year. The incidence increases slightly in Tehran with 1.6/100 000 per year. Also, it was reported that the disease is more prevalent in females and the age of onset was a bit lower than classically reported. About 6.2% eventually die of the disease (Chams-Davatchi et al., 2005).

In Shiraz, south-western Iran the incidence of pemphigus was 0.67/100 000 per year, with data collected from 1991 to 2000. The mean age of incidence was 38 years and, again, more prevalence in women. PV was the most frequent, 87.7% and, next, PF with 9%. It was verified that the first manifestation mostly occurred during winter, in about 30.8% of the patients (Salmanpour et al., 2006). A cohort from Isfahan was also evaluated, with 188 PV patients, 72 men and 116 women, from 1994 to 2004. It was determined an annual incidence rate of 5/100 000 and an age of

onset of 41.1 years old. Also, in 74% of the patients the mucosal involvement was the initial presentation and in the remaining 26% the skin was firstly affected by disease. Once again, women hold the largest incidence. In the course of the study, 9 patients died due to pneumonia and septicaemia (Asilian et al., 2006). In central part of Iran, in Yazd province, a cohort with 89 pemphigus patients, comprising 44.9% male and 55.1% female, from 1996 to 2006, and the incidence was determined to be 0.98/100 000 and the mean age of onset was 44.9 years. Once again, the most common form of pemphigus is PV with a probability 3.4 times higher than PF (Noorbala, et al., 2012). There is a higher incidence of PV in Iran, when compared to other regions of the world and, also, the mean age of onset is earlier (Asilian et al., 2006).

A retrospective study with 122 BP patients, from 1987 to 2007, was made to determine clinical and demographic characteristics of BP in Iranian patients. The mean age of onset was 65 years old, and the cohort comprised 35.2% males and 64.8% females. About 97.5% had cutaneous bullae, 27% had oral lesions and 30.3% had eosinophilia (Esmaili et al., 2012). Similar data about clinical phenotype in Iranian patients have already been reported (Daneshpazhooh et al., 2007).

**c. India**

A recent study gives us to know the reality of clinic histopathology patterns in eastern India, with a total 41 patients, over a period of a year. Of the 41 patients, 32 cases have PV (78%) and 8 cases with PF (19.51%). Most of the patients were between 41 and 60 years, and about 26.26% of the cases were above 60 years. Also, a female predominance was demonstrated in the PV cases, however, in PF cases, the sex ration was equal. In PV, initial lesions involve mucous membranes in 40.62% of the cases. Moreover, the eventual mucosal involvement was seen in (63.41%). It is possible to notice a change in PV disease development, which could represent a paradigm shift in this study population. In PF cases, 25% had both mucosal and skin involvement, and 75% of the cases had only skin manifestations. Again, when we are referring to PV, 20 cases (62.5%) demonstrated intra-epidermal suprabasal vesicles and about 5 cases (15.62%) demonstrated mid-epidermal vesicles. Twenty-nine cases showed acantholysis, which represent 90.62%. Cases with pure mucosal cases had no blister development. Twenty-three cases (53.5%) demonstrated inflammatory infiltrate in bulla cavity, and row of tombstone appearance was present in 17 cases (53.12%). Concerning the eight PF cases, seven cases (87.50%) showed acantholysis. Half of the cases had inflammatory infiltrate in bulla cavity. The DIF analysis revealed that 19 of the 32 PV cases had intra-epidermal deposition of intercellular deposition of intercellular IgG and C3 and 13 of the 32 had IgG deposition. In the PV cases, 87, 5% revealed antibody deposition in epidermis and 9.7% in the lower epidermis. Also, 71.87% cases had moderate strength of deposit. In PF group, 75% the antibody deposition occurred in the upper epidermis and 87.5% had a higher strength of deposit. For instance, the DIF intensity had a weak correlation with disease severity/activity scores. In PF, DIF can be used to evaluate the extent of skin involvement (Chowdhury et al., 2016).

**d. Thailand**

Between 1991 and 2009, a study comprised 58 BP patients to determine clinical characteristics in Thai patients. The cohort was obtained in Bangkok. The mean age of onset in these patients was 69.3 years with a higher prevalence in women. Mucosal involvement was seen in 15% of all patients and 38.9% had eosinophilia. It was also reported high inicidence of hypertension (41.4%), diabetes mellitus (19%) and cardiovascular disorders (24.1%) in these patients (Kulthanan et al., 2011).

**e. Taiwan**

The stimative of incidence of pemphigus and mortality rates in Taiwan revealed in cohort of 853 patients newly diagnosed from 2002 to 2009, in which 42.9% were men and 57.1% were women. The incidence was 4.7 per million per year and 88 patients died during the follow up period of 3.8 years, maily due to pneumonia, septicemia, cardiovascular disease and peptic ulcer disease. Also, the mean age of onset was 52.5 years old. It was also possible to determine that the incidence was higher in women (Huang et al., 2012).

## 10.4. America

**a. Brazil**

During a period of 2003 to 2010, records of hospitalized patients with PV and PF were collected from the Dermatology Service of Hospital Fundação Santa Casa de Mesericórdia do Pará, Belém, Northern Brazil. A total of 20 pemphigus cases were analysed, in which 8 were PF and 12 were PV. Male patients were predominant in PF, with 75%, whereas, females were predominant in PV cases, 66.7%. Three patients died (25%) (Pires et al., 2014). A study made in 2011 noticed that in the northeast region of the state of São Paulo, Brazil, where PF is endemic, the annual incidence is decreasing, whereas the incidence of PV in increasing. This study used a wide cohort with 163 cases of PF and 103 cases of PV, over 21 years, from 1988 to 2008. This incidence of PV had a mean age of 41.5 years old and the mean age of incidence of PF was 32.1 years old. Since 1998 that PV has been exceeding the incidence of PF (Gonçalves et al., 2011).

Usually PF (FS) occurs in Brazilian states localized between 45º and 60 º west longitude and 5º to 25º south latitude, and an altitude between 500 and 800 m (Culton et al., 2008). Ameridian reservation of Limão Verde is located in Mato Grosso do Sul and is home of 1351 members of the Terena tribe of Ameridians and is also an active FS focus with 3% of disease prevalence (Hans-Filho et al., 1996).

**b. Colombia**

Between 1992 and 2001, it was found 130 patients with PV (FS) in El Bagre surroundings, Colombia. About 90% of the cases started in the municipality of El Bagre (90%) and the remaining 10% in the neighboring rural areas. The majority was men (95.4%) with a mean age of onset of 50 years; the remaining 4.6% were women in post menopause. Also, 98% were illiterate and poor and had outdoors activities like farming. It is also important to note that they live without sewage and water systems, but all have good personal and home hygiene (Abrèu-Velez et al., 2003).

**c. USA**

In Hartford County, Connecticut, a study provided data that estimated the incidence of pemphigus, overall, was 0.42 cases per 100 000. Also Jewish adult population was investigated, revealing an incidence rate of 3.2 cases per 100 000, confirming that Jewish inhabitants had a higher risk of pemphigus than the general population (Simon et al., 1980).

## 10.5. Concluding Remarks

This final chapter helps understanding how these ABD spread around the globe. There is a pattern, where it is possible to see that in Africa, America and Asia, the incidence of PV and PF predominates. Whereas in Europe there a major incidence of BP and PV. These differences found in all continents are maybe due different cultures, populations' quality of life, economic and hygiene factors. Or maybe it is possible to infer that the genetic factor is also crucial. And this conclusion again brings us back to the question that it is necessary further genetic and epidemiologic studies to try to bridge this gap.

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# Final Conclusion

By the time I finished this dissertation I was sure about the inconsistencies that this issue entails.

This review results from the research of about 8% of all PubMed data, available about the subject. And still it seems that urge further research. It is necessary to fully and truly understand the exact pathway that causes blistering in pemphigoid and pemphigus, although we are on the right path. There are many coherent and accurate explanations, however, I feel that a mechanism that integrates all theories and tells us with certain what happens in molecular terms, is missing.

I could notice that, in the past few years, there has been a huge effort in order to find a better therapy for these patients, with fewer side effects and, preferably, one that doesn't include corticosteroids. It urges the need to design a novel and more specific therapeutic strategies that could counteract the chronic morbidity and mortality. It is necessary to know more about these ABD. And it may be necessary to begin by deepening the interference of triggers in these diseases.

Even when we talk about the diagnosis of these diseases we cannot say that accurating. Some patients are misdiagnosed, which delays the therapy.

The associated diseases are also a very important topic since it could lead us to understand how ABD behaves and how it really interferes with our organism, besides the skin. There are many diseased that have been correlated with these ABD.

It also could be interesting if the patients could be screened for genetic abnormalities, since it is very possible that these ABD have something to do with some somatic mutations. And this field may be transversal to all other areas of study.

If we evaluate the epidemiologic data alone it may not show the real impact of these diseases worldwide but if, for example, we do the math for the Swiss population, every year they have approximately 100 new BP cases. And in France an astonish number of about 1300 new BP cases per year.

Essentially, the purpose of this review, through an exhaustive research, was to guide us in the sense of perceiving what remains to be done and what is lacking for the patients. It became extremely important to be aware of the condition of these patients. The three ABD addressed in this dissertation are serious and dangerous conditions that put the patients at risk. Also, and beside the physical part, the daily life and psychosocial well-being are too affected. In the course of this exhaustive review, I only found one recent study about the quality of life of the patients, from Kouris et al., 2016. And only more recently there has been more research on more effective and less painful and deadly therapies for patients, for example. Only more recently there seems to be more awareness of what these diseases actually do to patients.

Perhaps, if we look more at the prism of the patient and what has been done up to now and even the price to pay for therapy with corticosteroids, we will be able to see that so much work that has yet to be done. It is scary that many patients still die not because of the disease itself but due to the corticosteroid therapy.

In the present year, Ahmed et al. (2016) published an article with the main concern for the future, suggesting more molecular pathways that still need to be worked, new theories and new possibilities.

Also, in the end of each chapter I careful sublimate some interesting points that could help to clarify better what could happen in each field of these diseases.

As I said in the beginning of this dissertation, this review was born not only of the desire to complete my master's degree but also of the desire to better understand how much is still lacking about these autoimmune blistering diseases and how can we help the patients who are suffering also from the main therapy. With this review it was also possible to see that there are great researchers, doctors, professors mainly dedicated to the theme, active in the study of these diseases. And for this same reason my bibliography is so extensive. And as it is perceived there is no focus on the mechanism, this involves many molecules and many alternative ways, so it is difficult to be targeted, which leads us to ramble on the subject, over all the chapters I have spoken.

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